

A Study of the Involvement of Extracellular Pathogenesis-Related Proteins in the Defence Response of Roses to Pathogens

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ABSTRACT

Acquired disease resistance can be induced in rose by acibenzolar-S-methyl (BTH), a novel synthetic chemical which has been shown to induce a broad-spectrum disease resistance in many plant species. BTH was applied by dipping whole *in vitro* rose shoots into the chemical at different concentrations for a few seconds before returning them to shoot growth medium. Four days later the shoots were challenge inoculated *in vitro* with *Diplocarpon rosae* or *Agrobacterium tumefaciens* under otherwise aseptic conditions. Pretreatment of rose shoots with BTH led to resistance to *D. rosae* and *A. tumefaciens* by significantly reducing the disease severity of blackspot and frequency of crown gall formation and gall sizes, respectively. The induced disease resistance by BTH may provide a novel approach to protect rose plants from pathogen infection in the field.

The biochemical mechanism behind the induced resistance was investigated based on analyses of protein changes in the intercellular spaces of rose leaves by SDS-PAGE, 2D-PAGE and western blotting of 1D- or 2D-gels against antisera of the four major classes of tobacco PR proteins. The results indicate that BTH-mediated enhanced resistance in rose was accompanied by the induction and accumulation of a set of extracellular proteins, including PR-1, PR-2, PR-3 and PR-5 proteins. Most of the extracellular proteins activated by BTH were also induced and found to accumulate in leaves upon infection with *D. rosae*. However, their accumulation was much more pronounced in BTH-pretreated leaves than in water-pretreated leaves upon a challenge inoculation with *D. rosae*. The induction and accumulation of PR-2 and PR-3 proteins in the intercellular spaces of BTH-treated leaves or BTH-pretreated leaves followed by infection with the pathogen was further confirmed by assays of β -1,3-glucanase and chitinase activities. All these results demonstrate that extracellular

PR proteins were involved in BTH-induced disease resistance in rose. However, not all isoforms of the PR proteins seemed to be associated with rose disease resistance based on western blots and native PAGE with specific staining of β -1,3-glucanase and chitinase activities. Some isoforms of the PR proteins such as 15 kDa PR-1, 36 and 37 kDa PR-2 proteins may be more important in the expression of resistance, whereas some isoforms may be involved in the growth and development of rose shoots. In conclusion, here it has been demonstrated that BTH is an effective inducer of PR proteins and acquired disease resistance in rose.

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ABBREVIATIONS

2D-PAGE	two-dimensional polyacrylamide gel electrophoresis
ANOVA	analysis of variance
BA	Benzyladenine
BCIP	5-bromo-4-chloro-indolyl-phosphate
Bis	N-N'-methylene-bis-acrylamide
BSA	bovine serum albumin
BTH	benzo(1,2,3)thiadiazole-7-carbothioic acid S-methyl ester
°C	degrees Celsius
cDNA	complimentary deoxyribose nucleic acid
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate)
cm	centimetre(s)
CM-chitin-RBV	carboxy methyl-chitin-Remazol Brilliant Violet
dH₂O	distilled water
EDTA	ethylenediaminetetraacetic acid
FW	fresh weight
g	gram(s)
GUS	β-glucuronidase
hr	hour(s)
HR	hypersensitive reaction
l	litre(s)
ICF	Intercellular fluid
IEF	isoelectric focusing
INA	2,6-dichloroisonicotinic acid

kDa	kilodalton
Mol	mole(s)
Min	minute(s)
mRNA	messenger ribonucleic acid
NBT	nitro blue tetrazolium
PAGE	polyacrylamide gel electrophoresis
PAL	phe ammonia-lyase
PBS	phosphate buffered saline
PDA	potato dextrose agar
pI	isoelectric point
PR	pathogenesis-related
PVDF	polyvinylidene difluoride
PVP	polyvinylpolypyrrolidone
SA	salicylic acid
SAR	systemic acquired resistance
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TBS	Tris buffered saline
TCA	trichloroacetic acid
TEMED	N-N-N'-N'-tetramethylethylene diamine
TL	thaumatin-like
TMV	tobacco mosaic virus
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
UV	Ultraviolet
X-Gluc	5-bromo-4-chloro-3-indoly glucuronide

Chapter 1

INTRODUCTION

1.1 General Introduction

The health of roses (*Rosa hybrida*), which are popular garden and cut flower plants worldwide, can be adversely affected by numerous diseases. The most important rose diseases are caused by fungi, such as powdery mildew and blackspot. These diseases seriously influence plant growth and saleability of cut rose flowers. In order to control these diseases, especially in the glasshouse, it has been necessary to use large amounts of fungicides that adversely affect the environment and raise the production costs (Horst 1983, Coyier 1985, Horst et al. 1992, Gullino and Garibaldi 1995). Besides, rose production has been constantly threatened by virus and bacterium infection. For example, rose mosaic caused by prunus necrotic ringspot virus (PNRV) and crown gall caused by *Agrobacterium tumefaciens* are extremely common rose diseases (Thomas 1982, Horst 1983, Coyier 1985, Gullino and Garibaldi 1995). Therefore, rose cultivars with improved resistance to pathogens and an understanding of the resistance mechanisms involved have become increasingly important.

Reaction of plants to phytopathogens (viruses, bacteria and fungi) has been the subject of active research. Plants defend themselves from pathogen or stress through activating a range of defence responses, such as hypersensitive response (HR), a rapid production of reactive oxygen species, enhancement of lignification and cell wall cross-linking, production of phytoalexins and the accumulation of large amounts of pathogenesis-related (PR) proteins (Collinge and Slusarenko 1987, Bowles 1990, Dixon et al. 1994, Mehdy 1994, Kuc' 1997). Among these defence responses, the

production of PR proteins is extensively studied in tobacco and a few other species. An intriguing possibility that has emerged recently, mainly from work on tobacco, is that PR proteins might be an important part of the natural defence mechanisms (Van Loon 1985, Bol et al. 1990, Linthorst 1991, White and Antoniw 1991, Stintzi et al. 1993).

Upon infection with a pathogen, plants hypersensitively produce necrotic lesions at the infection sites, which prevent further spread of the pathogen and subsequent pathogenic attack. This resistant reaction is accompanied by the induction and accumulation of large amounts of PR proteins in the uninfected parts of the plant that develop a resistance against the same or other pathogens. This phenomenon is termed systemic acquired resistance (SAR) (Neuenschwander et al. 1996, Ryals et al. 1996, Lucas 1999). Induced resistance is accompanied by the expression of a set of so called SAR genes that can be broadly classified as those encoding PR proteins (Ryals et al. 1996, Uknes et al. 1996). The strong correlation between acquired resistance and PR gene expression has led to the hypothesis that PR proteins are directly involved in plant defence response to pathogens (Linthorst 1991, Stintzi et al. 1993, Van Loon 1997). Furthermore, it was found that many PR proteins have direct antimicrobial activity *in vitro* (Mauch et al. 1988, Leah et al. 1991, Huynh et al. 1992, Niderman et al. 1995, Koiwa et al. 1997) and transgenic plants expressing increased level of PR genes show increased resistance to pathogen infection (Brogliè et al. 1991, Zhu et al. 1994, Niderman et al. 1995, Tabei et al. 1998).

Induced resistance, based on natural defence mechanisms of plants, is a very promising alternative approach to control plant diseases. This approach is effective and economical and would reduce the dependence on agrochemicals (Lucas 1999). Induced resistance has been described in a variety of plant species after infection by various pathogens or treatment with natural or synthetic chemicals (Kessmann et al. 1994, Neuenschwander et al. 1996, Schneider et al. 1996). Up to now, only one paper was published on the induced resistance to *Sphaerotheca pannosa* by 2,6-dichloroisonicotinic acid (INA) in rose (Hijwegen et al. 1996). Recently, benzo(1,2,3)thiadiazole-7-carbothioic acid S-methyl ester (BTH), a synthetic resistance-inducing chemical, has received much attention. This chemical can provide

good protection against different crop diseases. The BTH-induced resistance is accompanied by the systemic expression of a set of PR genes in a wide variety of crops (Friedrich et al. 1996, Görlach et al. 1996, Lawton et al. 1996, Siegrist et al. 1997, Inbar et al. 1998). BTH has therefore been considered as a potential resistance-inducing agent for agricultural disease control in the field (Görlach et al. 1996, Uknes et al. 1996). However, the potential of BTH for disease protection in ornamental plants remains to be explored.

Rose is one of the most important flowers in the world, but yet little is known about its natural disease defence systems, especially PR proteins. Therefore, whether BTH can induce acquired resistance against rose diseases in rose plants and whether the induced resistance is also associated with the accumulation of a number of PR proteins are of particular interest in this study.

1.2 Pathogenesis-related proteins

Upon infection with a pathogen, many plants develop disease symptoms and are accompanied with the synthesis of some new proteins. The occurrence of these new proteins is not pathogen-specific, but is determined by the type of reaction displayed by the host plant, indicating that they are of host origin. Since the proteins are induced under specific pathological conditions, they have been named pathogenesis-related proteins or PR proteins (PRs) (Van Loon 1985). PR proteins were initially found to be acidic proteins and secreted into the intercellular spaces of leaves. Therefore, PR proteins were defined as a group of acidic proteins found in the extracellular spaces of leaves (Van Loon and Van Kammen 1970, Van Loon 1985). Later, basic PR proteins were identified at relatively low levels in the vacuole (Carr and Klessig 1989, Bol et al. 1990). Thus, the definition of PR proteins has been broadened to include intra- and extracellularly localised induced proteins (Bowles 1990). It was found further that PR proteins are induced not only by pathogen attack, but also by treatment with certain chemicals, or other types of stresses (Linthorst 1991, Stintzi et al. 1993). Therefore, PR proteins have now been redefined as plant proteins that are induced in pathological or related situations (Van Loon et al. 1994).

Over the last decades PR proteins have been studied extensively in order to determine their function in the plant disease resistance (Linthorst 1991). It was assumed that the accumulation of PR proteins is an important feature of plant defence response upon infection by pathogens or other stress-inducing agents in various plant species (Van Loon et al. 1987, Nasser et al. 1988, White and Antoniw 1991, Lawton et al. 1993). Support for this was given by the findings that the accumulation of PR proteins is closely correlated with the disease resistance of plants and most of the PR proteins have been identified to have antimicrobial activity *in vitro* (Linthorst 1991, White and Antoniw 1991, Stintzi et al. 1993, Van Loon 1997). Moreover, transgenic plants that overexpress or underexpress PR proteins have been generated by different research groups. The results indicated that transgenic plants constitutively expressing PR proteins showed increased disease resistance (Broglie et al. 1991, Alexander et al. 1993, Zhu et al. 1994, Marchant et al. 1998). In contrast, reducing the expression of the β -1,3-glucanase gene by generating antisense plants resulted in a lower level of resistance in one of the transgenic tobacco lines (Lusso and Kuc' 1996).

Since the tobacco PR proteins are most widely studied and best characterised, most information discussed in this chapter is based on the tobacco PR proteins.

1.2.1 Characteristics

PR proteins are relatively stable at low pH and remain soluble at pH 3, while most other plant proteins are denatured under these acidic conditions (Van Loon and Van Kammen 1970, Antoniw and White 1983). The second remarkable property that distinguishes PR proteins from normal plant proteins is their resistance to a range of proteases (Van Loon 1983). They are also characterised by their low molecular weights (Van Loon 1985); and their extracellular localisation (Parent and Asselin 1984, Carr et al. 1987). However, basic homologues to some of the acidic PR proteins have been identified (Carr and Klessig 1989) and they accumulate in the vacuoles (Bol et al. 1990). In addition, some PR proteins possess extreme isoelectric points (Van Loon 1985).

1.2.2 Occurrence and induction

PR proteins were first detected in tobacco plants reacting hypersensitively to tobacco mosaic virus (TMV) infection in early 1970s (Van Loon and Van Kammen 1970). Since then, PR proteins have been identified in at least nine families including more than 30 different plant species following infection with different pathogens (viruses, viroids, bacteria or fungi) (Mohamed and Sehgal 1997). Usually, the most effective inducers of PR proteins are pathogens that cause a necrotic reaction resulting in the accumulation of the largest amounts of PR proteins in tissue adjacent to the necrotic areas. However, some pathogens can induce large amounts of PR proteins without causing necrosis (Van Loon 1985, Carr and Klessig 1989, Bol et al. 1990, Bowles 1990, Linthorst 1991, Stintzi et al. 1993). The accumulation of PR proteins in infected tissue is often observed in incompatible plant-pathogen interactions, although such a process is not restricted to resistant cultivars (Linthorst 1991). However, early and/or increased accumulation of PR proteins was found in resistant cultivars following pathogen attack (Daugrois et al. 1990, Lawrence et al. 1996, Roulin et al. 1997).

PR proteins can also be induced by treatment with chemicals such as DL-3-amino-n-butanoic acid (Asselin et al. 1985), ethephon (Vera and Conejero 1990), probenazole (Iwata et al. 1980), and salicylic acid (Yalpani et al. 1993), or environmental stress such as UV light (Jung et al. 1995) and air pollutants (Ernst et al. 1992). However, PR proteins have also been shown to accumulate in healthy plants during flowering and senescence (Fraser 1981, Lotan et al. 1989, Smart 1994).

The acidic PR proteins are induced mostly upon pathogen infection and by salicylic acid treatment (Linthorst 1991, Ryals et al. 1996), whereas basic proteins are induced by ethylene, methyljasmonate and wounding (Xu et al. 1994). The basic proteins are also expressed in a tissue-specific and developmentally controlled manner in leaves, roots and floral parts of plants (Linthorst 1991).

1.2.3 Classification

The four PR proteins designated PR-1a, -1b, -1c and PR-2, large amounts of which accumulating in infected tobacco leaves, were first discovered and characterised (Stintzi et al. 1993). Subsequently, a number of additional PR proteins had been reported. These additional components were named as PR-N, -O, -P, -Q, -R and PR-S in order of decreasing mobility in native polyacrylamide gels (Van Loon et al. 1987). With the purification of these proteins, cDNAs and gene(s) cloned, their antibodies and many sequence data became available. Numerous observations were also made in other plants where both extracellular and vacuolar forms of PR proteins were identified (Antoniw and White 1983, Linthorst 1991, White and Antoniow 1991, Stintzi et al. 1993). To enable an easier comparison of related PR proteins between different plant species, PR proteins were originally grouped into five families (PR-1 to PR-5) on the basis of biological function, sequence similarity and immunological relationship of tobacco PR proteins (Van Loon et al. 1987, Carr and Klessig 1989). The current classification system places PR proteins into 11 families according to their amino acid sequences, serological relationships and/or enzymatic or biological activities. In addition to 5 well-studied families in tobacco, a further 6 families (PR-6 to PR-11) were proposed to be classified as PR proteins (Van Loon et al. 1994). However, not all families are represented in any plant species. Each family may consist of several members (Van Loon 1997). With the discovery of additional pathogen-induced proteins, three antimicrobial peptides have now been considered as additional families of PR proteins, respectively (Van Loon and Van Strien 1999).

1.2.3.1 PR-1 proteins

PR-1 family in tobacco consists of three acidic proteins (PR-1a, PR-1b and PR-1c) and two basic proteins (designated cluster G-protein and PRB-1b) (Jamet and Fritig 1986, Bryngelsson et al. 1994). PR-1a, -1b and -1c proteins were the first discovered and purified PR proteins. They are shown to have similar molecular weights (approximately 15-17 kDa), amino acid compositions and close serological relationships. They accumulate extracellularly and predominantly in xylem elements of infected leaves (Parent and Asselin 1984, Carr et al. 1987, Stintzi et al. 1993). The

tobacco basic PR-1 proteins were found to have an intracellular localisation and show about 65% amino acid similarity to the acidic PR-1 proteins and contain an extra domain absent in acidic-type isoforms (Cornelissen et al. 1987, Eyal et al. 1992, Sessa et al. 1995).

In tobacco, expression of basic and acidic isoforms of PR-1 proteins is differentially regulated. The basic PR-1 gene is activated by the plant hormone ethylene and shows constitutive expression in phloem tissues (Eyal et al. 1992). Conversely, acidic PR-1 genes are induced during the HR and the development of SAR or salicylic acid treatment (Linthorst 1991). The acidic PR-1 proteins, the most abundant members of the tobacco PR proteins, are highly induced in infected tissue and accumulate up to 1-2% of total soluble leaf protein content, suggesting an important role for this family of proteins in disease resistance (Alexander et al. 1993).

PR-1-like proteins have been identified in many plant species upon pathogen infection or in response to chemical treatments and show a high degree of amino acid identity to the tobacco PR-1 proteins (Linthorst 1991, Stintzi et al. 1993). Interestingly, PR-1-like proteins have also been identified in mammal and humans (Kitajima and Sato 1999). The widespread occurrence of PR-1-like proteins also suggests that they play an essential role in response to stress (Linthorst 1991).

1.2.3.2 PR-2 proteins

The tobacco PR-2 proteins were initially induced upon TMV infection (Gianinazzi et al. 1970, Van Loon and Van Kammen 1970) and have since been found that they are not related to the members of the PR-1 family (Antoniw and White 1983). Later, a purified PR-2 protein was found to have β -1,3-glucanase activity (Kauffmann et al. 1987). β -1,3-glucanases (EC 3.2.1.39) hydrolyse laminarin. Both acidic and basic proteins with β -1,3-glucanase activity have been detected in tobacco (Linthorst 1991). So far at least three structural classes of β -1,3-glucanases have been identified (Stintzi et al. 1993). The first class consists of at least three basic proteins that accumulate predominately in the vacuole. These enzymes are encoded by a small gene family and cDNA clones encoding three isoforms have been isolated (Shinshi et al. 1988, Vögeli-Lange et al. 1994). They are induced by either pathogen infection (Kauffmann et al.

1987, Vogeli-Lange et al. 1988, Ward et al. 1991a) or ethylene treatment (Felix and Meins 1987), and exhibit developmental regulation (Felix and Meins 1986). The second class of β -1,3-glucanases consists of extracellular acidic proteins PR-2, PR-N, PR-O and two glycoproteins localised in the style of the flower (Kauffmann et al. 1987, Linthorst 1991, Ward et al. 1991a). They are regulated by both pathogen infection and developmental signals (Boller 1985). However, the flower glucanase genes are not induced in the leaves by infection or salicylate treatment (Ori et al. 1990). The third class consists of only one enzyme PR-Q'. It is also an extracellular acidic protein, but differs substantially from both the basic vacuolar glucanase and the acidic extracellular enzymes with 50 to 60% identity at amino acid level (Payne et al. 1990).

β -1,3-glucanases have been identified in many higher plants in response to pathogen infection or chemical treatments (Stintzi et al. 1993). It was also found that basic β -1,3-glucanases could be expressed constitutively to high levels in roots and flowers (Linthorst 1991). In addition, the expression of β -1,3-glucanases at the mRNA level was regulated by the plant hormones auxin, cytokinin, and ethylene besides pathogen infection (Cabello et al. 1994).

1.2.3.3 Chitinase

The groups of PR-3, PR-8, PR-11 proteins all comprise chitinase enzymes (Van Loon et al. 1994). Chitinases catalyse the hydrolysis of chitin which is a major component of the exoskeleton of insects and of cell walls of most fungi (Boller 1987, Punja and Zhang 1993). This enzyme was initially found to be induced following TMV infection in tobacco (Legrand et al. 1987), but is now known to be induced by a wide variety of both biotic and abiotic elicitors (Linthorst 1991, Stintzi et al. 1993, Graham and Sticklen 1994). Induction of chitinase also occurs in response to developmental and hormonal regulation (Shinshi et al. 1987). Chitinases have been reported from over 41 monocotyledonous and dicotyledonous plants (Punja and Zhang 1993) and occur in widely different tissues such as maize seeds (Cordero et al. 1994), cucumber hypocotyls (Kästner et al. 1998), tobacco roots (Tahiri-Alaoui et al. 1990), pepper

stems (Hwang et al. 1997), tomato leaves (Lawrence et al. 1996), petunia flower tissues (Leung 1992), and fruits of kiwifruit (Wurms et al. 1997).

Chitinases are classified into five structural classes based on amino acid sequences and subcellular topology (Collinge et al. 1993, Punja and Zhang 1993, Stintzi et al. 1993, Melchers et al. 1994). Class I chitinases are basic forms and occur in vacuoles. The enzymes contain an N-terminal cysteine-rich domain, which supposedly functions in chitin binding. Furthermore, a hinge domain was assigned, and a C-terminal vacuolar targeting sequence was identified (Broglie et al. 1986, Shinshi et al. 1990, Zhu and Lamb 1991). In general, class II chitinases are acidic and extracellular enzymes, including PR-P and PR-Q. The sequences of these proteins are similar to those of class I chitinases, but lack the N-terminal cysteine-rich domain, the hinge region, and the C-terminal extension (Linthorst et al. 1990, Payne et al. 1990). Class III chitinases are characterised by bifunctional, lysozyme activity and have no sequence similarity to class I or class II chitinases (Mettraux et al. 1989). They include the acidic and basic extracellular chitinases (Mettraux et al. 1989, Lawton et al. 1992, Punja and Zhang 1993). Class IV chitinases resemble the class I chitinases in containing a cysteine-rich domain and a conserved main structure. However, they are serologically distinguishable from class I chitinases and about 50 amino acid residues smaller due to four deletions (Collinge et al. 1993, Vidhyasekaran 1997). Class V chitinases show no sequence similarity to chitinases classes I-IV. They share significant homology with some bacterial exochitinases, but are endochitinases lacking detectable exochitinase activity (Melchers et al. 1994). The class I, II and IV chitinases were grouped into PR-3 family. According to Van Loon's (1994) new classification of PR proteins, the class III chitinases that possess lysozyme activities and strongly differ in sequence and/or substrate preference from the class I and II chitinases are classified as PR-8 family, while the tobacco class V chitinases are members of the PR-11 family.

1.2.3.4 PR-4 proteins

PR-4 proteins have been reported in tobacco and tomato (Van Loon et al. 1987, Joosten et al. 1990) and are homologous to the carboxy-terminal domains of hevein

and potato Win-1 and Win-2 proteins, which showed affinity for chitin (Friedrich et al. 1991). However, PR-4 proteins lack the amino-terminal Cys-rich lectin domain that can bind to chitin. Class I PR-4 protein of tobacco is localised intracellularly and contains a C-terminal domain. Class II PR-4 proteins include four acidic and extracellular proteins, r1, r2, s1, and s2 named by Kauffmann et al. (1990). They are serologically related to each other and homologous to class I PR-4 protein. However, the biological function or activity of these proteins is not clear (Linthorst 1991, Stintzi et al. 1993).

1.2.3.5 PR-5 proteins

PR-5 proteins have amino acid sequences, molecular weights, circular dichroism spectra similar to those of thaumatin which is an intensely sweet protein isolated from the fruit of the West African shrub *Thaumatococcus danielli* (Vidhyasekaran 1997). Permatins, osmotins, and zeamatins, which are proteins of different origins, have been shown to have amino acid sequences similar to that of thaumatin, and are thus called thaumatin-like (TL) proteins (Koiwa et al. 1997). Acidic PR-5 proteins localised in the apoplastic spaces have been detected in several crops. This class includes two tobacco PR proteins which were initially named PR-R and PR-S (Stintzi et al. 1993). The basic PR-5 proteins have been identified as osmotins, which accumulate in ethylene-treated leaves and in the salt-adapted cells. They are localised in vacuoles or cytoplasmic vesicles (Singh et al. 1987, Stintzi et al. 1991). A neutral PR-5 protein, PR-5d, occurs in roots and in cultured cells (Koiwa et al. 1997). All PR-5 proteins are immunologically related (Pierpoint et al. 1992) and show 65% amino acid sequences homology to a maize protein that is a bifunctional inhibitor of α -amylase and protease of insects (Richardson et al. 1987). These PR-5 proteins have been induced by pathogen infection, as well as numerous developmental, hormonal, and environmental signals (Linthorst 1991, Stintzi et al. 1993, Pressey 1997).

1.2.3.6 Peroxidase

Peroxidases (E.C. 1.11.1.7) are enzymes related to physiological changes in plant-pathogen interaction. The tobacco “ligninforming peroxidase” is classified as a member of PR-9 family (Van Loon et al. 1994). The peroxidase has been considered

as a PR protein based on its characteristics and because it is only expressed in hypersensitively reacting leaves (Stintzi et al. 1993). In tomato, two anionic peroxidases are expressed in response to pathogen, wounding (Mohan et al. 1993) and elicitor treatment (Mohan and Kolattukudy 1990). Parent and Asselin (1987) showed that the PR proteins of potato include peroxidases. 2,6-Dichloroisonicotinic acid as an elicitor of acquired resistance induces the accumulation of peroxidase isoforms in barley leaves (Kogel et al. 1994). The activity of cell wall bound peroxidase in tomato hypocotyls is also enhanced by treatment with 4-hydroxybenzoic hydrazide, salicylic hydrazide and 2-furoic acid (Miyazawa et al. 1998). The basic isoform of peroxidase is not only induced by ethylene, but also expressed in response to wounding of tissue and treatment with salicylate in azuki bean leaves (Ishige et al. 1993b). In addition, ethylene-induced cucumber peroxidase cDNA sequences have also been obtained (Linthorst 1991). In rice, each peroxidase gene displays unique regulatory behaviour in response to different environmental stimuli (Chittoor et al. 1997).

1.2.3.7 Other PR proteins

Tomato proteinase inhibitor I, tomato endoproteinase and parsley “ribonuclease-like” are classified into PR-6, PR-7 and PR-10 families, respectively (Van Loon et al. 1994). These proteins are also found in other species. They are induced by pathogens and by application of elicitors (Ryan 1981, Van der Wilden et al. 1983). Recently, plant defensins (radish Rs-AFP3), thionins (*Arabidopsis* THI2.1), and lectins (barley LTP4) are recently classified into PR-12, PR-13, and PR-14 families, respectively (Van Loon and Van Strien 1999). These proteins commonly occur in storage organs, such as seeds and tubers, but may also be induced in leaves after pathogen infection (Broekaert et al. 1995).

1.3 Association of PR proteins with induced resistance

Induced resistance is the phenomenon that a plant, once appropriately stimulated, exhibits an increased resistance against a broad spectrum of pathogens without

alterations of the plant genome (Van Loon 1997). In recent years, induced resistance has attracted a lot of attention both in applied agriculture and basic research.

1.3.1 Hypersensitive response (HR)

In response to pathogen attack, a plant rapidly produces a necrotic lesion at the infected site, which restricts further growth and spread of the pathogen and subsequent pathogen infection. This resistant reaction is designated as the hypersensitive response. The hypersensitive response to a pathogen infection is considered as a very common reaction in plants and one of the most efficient defence mechanism as well (Hammond-Kosack and Jones 1996). It is believed that HR is genetically programmed and confers disease resistance due to the recognition and interaction of biochemical components from both the pathogen and host (Hammond-Kosack and Jones 1996, Jackson and Taylor 1996). This response involves the rapid, localised plant cell death, and is accompanied with a dramatic set of metabolic changes locally or systemically in the plants, such as synthesis of PR proteins which have been postulated to play an important role in plant defence response. Larger amounts of PR proteins are present in tissue adjacent to the necrotic areas (Linthorst 1991, Stintzi et al. 1993). In addition, active oxygen species including superoxide and hydrogen peroxide may be generated during the hypersensitive response. The oxidants may function as protective agents directly, or function in cell wall cross-linking or as part of signalling mechanisms (Kuc' 1997). The activities of peroxidase and phenylalanine ammonia-lyase (PAL), deposition of lignin and phytoalexins are also increased during HR. Phytoalexins are low molecular weight compounds that inhibit the growth of microorganisms (Kuc' 1997). Peroxidase is involved in cross-linking extensin molecules and in the polymerisation of hydroxycinnamyl alcohols to form lignin (Hammerschmidt and Kuc 1982, Irving and Kuc' 1990, Dalisay and Kuc 1995b). Increased lignin deposition is believed to play a role in barricading the pathogen from invading the plant through physical exclusion (Hammerschmidt and Kuc 1982, Hammond-Kosack and Jones 1996). PAL is responsible for the conversion of phenylalanine to *trans*-cinnamic acid, a key intermediate in the pathway for production of lignin and salicylic acid (SA) (Hammond-Kosack and Jones 1996).

1.3.2 Systemic acquired resistance (SAR)

It was first shown nearly 40 years ago that tobacco plants previously infected by tobacco mosaic virus (TMV) had become resistant to a subsequent infection by TMV (Ross 1961a, Ross 1961b). Since then induced resistance has been an attractive research area (Schneider et al. 1996). Induced resistance can be either local or systemic. Local resistance may be induced in tissues adjacent to the infected or wounded sites. While the uninoculated parts of the plant may also exhibit an increased level of resistance with much smaller lesions and greater restriction to challenge inoculation by the same or other pathogens. This is termed systemic acquired resistance (Ryals et al. 1996, Van Loon 1997). For example, cucumber plants inoculated with the fungus *Colletotrichum lagenarium* resulted in SAR against a dozen diseases caused by fungal and bacterial as well as viral pathogens (Sticher et al. 1997).

SAR can be activated by a variety of biotic (bacteria, fungi and viruses) and abiotic elicitors. In addition to pathogenic microorganisms, nonpathogenic rhizobacteria have also been demonstrated to induce SAR in several plant species (Van Loon 1997). Abiotic agents include natural products and synthetic compounds that have been found to induce resistance without causing any visible symptoms (Kessmann et al. 1994). Recently, two synthetic compounds (INA and BTH) have attracted much attention (see later). What is clear is that induced resistance results from the plant's own defence mechanisms to restrict pathogen development.

SAR has been demonstrated in at least 20 plant species from at least six different plant families and is likely to be a ubiquitous higher plant defence response (Sticher et al. 1997, Lucas 1999). It has been postulated that the induction of resistance in uninoculated parts of an infected plant is due to the translocation of a hitherto unknown systemic signal produced at the site of primary infection. This signal could then activate the plant's own defence system against further pathogen attack. It is believed that salicylic acid plays an important signalling role in the resistance of many plants to pathogen infection (Sticher et al. 1997). Resistance is dependent on the high-level expression of SAR genes (Kessmann et al. 1994, Uknes et al. 1996). Therefore, a thorough understanding of SAR-establishing mechanism will lead to new disease

control approaches. These could include development of transgenic plants expressing a resistance gene for enhanced disease resistance, or new possibilities to manipulate key signalling components of SAR, or refining the search for novel chemical activators of disease resistance (Ryals et al. 1994).

1.3.2.1 SAR proteins or genes

A protein is classified as a SAR protein when its presence or activity correlates tightly with maintenance of resistance, and its corresponding gene as SAR gene (Neuenschwander et al. 1996). Systemic acquired resistance can be effective against a wide range of pathogens and is accompanied by the systemic expression of a set of so-called SAR genes in several plant species (Sticher et al. 1997). In both *Arabidopsis* and tobacco, the same set of SAR genes and the same spectrum of resistance were activated by both the chemical inducers such as SA, INA, and BTH and biological inducing agents (Ward et al. 1991b, Uknes et al. 1992, Friedrich et al. 1996, Lawton et al. 1996). Analysis of SAR proteins showed that many belong to the classes of PR proteins. Hence, the induction of these PR genes often serves as markers for the state of induced resistance (Ward et al. 1991b, Hunt and Ryals 1996, Van Loon 1997). However, not all PR proteins are equally induced during SAR. Many extracellular acidic but few basic PR proteins have been found to be expressed to high levels in the uninfected part of the plant (Van Loon and Antoniw 1982, Uknes et al. 1992). In tobacco, SAR genes are coordinately induced in the uninfected leaves of the plants. They are PR-1 (acidic, extracellular, function unknown); PR-2 (acidic, extracellular β -1,3-glucanase); PR-3 (acidic, extracellular chitinase); PR-4 (acidic, extracellular, unknown function); PR-5 (acidic, extracellular, thaumatin-like protein and bifunctional amylase/proteinase inhibitor of maize), PR-1 (basic form of PR-1), basic class III chitinase (structurally unrelated to PR-3); acidic class III chitinase (extracellular; approximately 60% identical to basic form); and PR-Q' (acidic, extracellular β -1,3-glucanase). In addition, recently, SAR 8.2 is also considered as a SAR protein. It is expressed at low level in untreated tissue and has also been found to be wound inducible (Ward et al. 1991b).

Several SAR genes are common in plants; however, differences in expression of specific genes are observed between species. In tobacco, PR-1 is most strongly induced and its presence was tightly correlated with SAR (Uknes et al. 1993), whereas in cucumber class III chitinase is the predominant SAR gene (Métraux and Boller 1986). In *Arabidopsis*, the SAR marker genes are PR-1, PR-2, and PR-5 (Uknes et al. 1992). Major and minor forms of PR-proteins expressed in various species are likely to be effective against different diseases (Schneider et al. 1996). Therefore, the presence of SAR proteins is consistent with a role of these proteins for maintenance of SAR.

1.3.2.2 Chemical induction of SAR

In addition to pathogen-induced SAR, several chemicals, which mimic the effect of pathogen infection or induce similar stresses, have been reported to induce SAR when applied to plants (Iwata et al. 1980, Cohen 1994, Kessmann et al. 1994, Schneider et al. 1996).

Chemical induction of systemic acquired resistance in plants is a promising approach in crop disease control. However, a chemical which is considered to be an activator of SAR should meet the following criteria: (1) The compound or its significant metabolites should not have antimicrobial activity *in vitro*. (2) It should induce resistance against the same spectrum of pathogens as in biologically activated SAR. (3) It should induce the expression of same marker genes as pathogen-activated SAR. Accordingly, only a few of chemicals qualify as activators of SAR (Kessmann et al. 1994).

Ethylene Ethylene is known as a plant stress hormone. It is involved not only in regulating the growth and development of plants (Yang and Hoffman 1984), but also in defence responses against pathogen attack (Boller 1982, Boller 1991). Pathogen infection can trigger the increased synthesis of ethylene when a cell undergoes necrosis and leads to the accumulation of PR proteins (Boller et al. 1983, Van Loon 1985, Liu et al. 1994). Exogenous application of ethylene in many plant species results in the induction of PR proteins (Van Loon 1977, Felix and Meins 1987, Vera and Conejero 1990, Brederode et al. 1991, Benčekrown et al. 1993). For example, ethylene enhanced β -1,3-glucanase and chitinase activities (Métraux and Boller 1986,

Bryngelsson et al. 1988, Takeuchi et al. 1990), as well as the transcription of the gene for the basic isoform of peroxidase (Ishige et al. 1993b). Therefore, ethylene may function as a physiological inducer of PR genes (Memelink et al. 1990) and as a systemic signal transduction molecule as it can induce systemic disease resistance (Vidhyasekaran 1997).

Salicylic acid (SA) Salicylic acid has long been known as an inducer of systemic acquired resistance (Ryals et al. 1994, Ryals et al. 1996, Durner et al. 1997). Pathogen infection results in significant accumulation of salicylic acid, which is correlated with disease resistance in cucumber (Malamy et al. 1990) and tobacco (Malamy et al. 1990). Exogenous application of salicylic acid (SA) to tobacco and *Arabidopsis* leads to the induction of acquired resistance to the same spectrum of pathogens as biological inducers of SAR, as well as expression of the SAR genes (Ward et al. 1991b, Uknes et al. 1992, Uknes et al. 1993, Yalpani et al. 1993). In addition, transgenic tobacco and *Arabidopsis* plants expressing the salicylate hydroxylase gene *NahG* unable to accumulate SA cannot activate SAR, suggesting that SA accumulation is required for establishment of SAR (Gaffney et al. 1993). In *Arabidopsis*, depletion of SA suppresses both SAR and gene-for-gene resistance (Delaney et al. 1994). These findings indicate that salicylic acid is a signalling molecule involved in both hypersensitive reaction and the induction of systemic resistance and is believed to play a key role of the signal transduction pathways (Ryals et al. 1994, Ryals et al. 1996, Durner et al. 1997).

2,6-dichloroisonicotinic acid (INA) 2,6-dichloroisonicotinic acid is a synthetic compound and only has weak antifungal activity *in vitro*. Recently, it has been shown that INA could induce broad-spectrum disease resistance against viruses, fungi and bacteria in many plant species (Ward et al. 1991b, Uknes et al. 1992, Kogel et al. 1994, Vernooij et al. 1995, Hijwegen et al. 1996). In tobacco, INA induces the resistance to the same spectrum of pathogens and the expression of the same set of SAR genes that are induced by either SA treatment or pathogens (Ward et al. 1991b). However, it does not induce SA accumulation during the induction of SAR in both tobacco and *Arabidopsis*, suggesting it might activate downstream of SAR signal transduction pathway (Vernooij et al. 1995). In other plants, some of SAR genes are also induced by INA (Uknes et al. 1992, Kogel et al. 1994).

Exogenous application of INA to leaves has been shown to induce SAR in treated and untreated tissues of the same plant (Vernooij et al. 1995). However, SA only induces SAR in the treated leaves (Enyedi et al. 1992, Malamy and Klessig 1992).

Benzo-1,2,3-thiadiazole-7-carbothioic acid S-methyl ester (BTH) More Recently, acibenzolar-S-methyl (benzo-1,2,3-thiadiazole-7-carbothioic acid S-methyl ester), which is referred to as BTH, a synthetic resistance-induced chemical, has received much attention. It is interesting to note that SA, INA and BTH have similar chemical structures, sharing an aromatic ring with a carboxyl group (Figure 1).

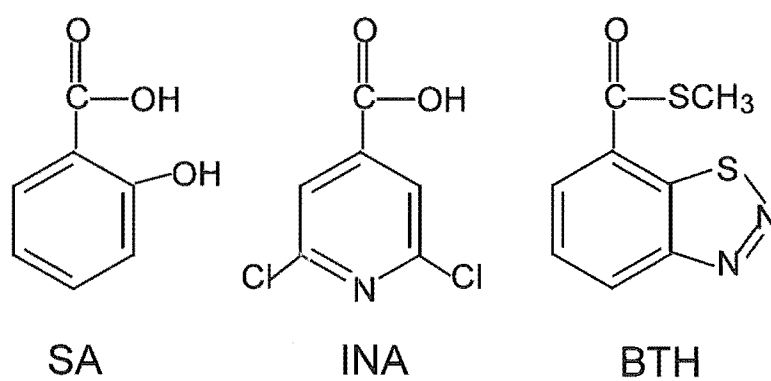


Figure 1.1 Chemical structures of three inducers of SAR.

BTH has been reported to induce broad-spectrum protection against viral, bacterial and fungal pathogens or reduce insect densities in a wide variety of crops (Table 1). However, it was found that BTH has no direct antifungal activity *in vitro*. In addition, its metabolites isolated from plants do not have any antimicrobial activity (Friedrich et al. 1996, Benhamou and Belanger 1998b, Ishii et al. 1999). These lines of evidence strongly suggest that BTH works via the activation of plant defence mechanisms. Like SA and INA, BTH induces the same spectrum of pathogen resistance and gene expression, as does pathogen infection (Friedrich et al. 1996, Lawton et al. 1996). Furthermore, it was found that BTH is a more effective inducer of defence gene expression than SA (Wendehenne et al. 1998). It has been suggested that BTH may act downstream of SA accumulation during the SAR signal transduction pathway as BTH does not stimulate SA biosynthesis and is able to induce SAR in *NahG* transgenic *Arabidopsis* plants (Lawton et al. 1996).

Table 1.1 Plants showing acquired disease resistance following BTH treatment

Plant species	Protection against	Induction of SAR genes	References
Tobacco	TMV <i>Cercospora nicotianae</i> <i>Peronospora tabacina</i> <i>Erwinia carotovora</i> <i>Phytophthora parasitica</i> <i>Pseudomonas syringae</i>	PR-1, PR-2, PR-3, PR-4, PR-5, PR-1 basic, Basic class III chitinase, Acidic class III chitinase, PR-Q', Basic glucanase, Basic chitinase, SAR 8.2, Acidic peroxidase	Friedrich et al. (1996)
Wheat	<i>Erysiphe graminis</i> <i>Puccinia recondita</i> <i>Septoria</i> spp. <i>Blumeria graminis</i> f. sp. <i>tritici</i>	WCI-1, WCI-2 (Lipoxygenase), WCI-3, WCI-4 (Cysteine proteinase), WCI-5, PR-1	Görlach et al. (1996) Stadnik and Buchenauer (1999)
<i>Arabidopsis</i>	Turnip crinkle virus <i>Pseudomonas syringae</i> <i>Peronospora parastica</i>	PR-1 PR-2 PR-5	Lawton et al. (1996)
Cucumber	<i>Pythium ultimum</i>	NM* Chitinase	Benhamou and Bélanger (1998) Kästner et al. (1998)
Green bean	<i>Uromyces aendiculatus</i> <i>Colletotrichum lindemuthianum</i> <i>Rhizoctonia solani</i> <i>Xanthomonas campestris</i>	Chitinase β -1,3-glucanase Peroxidase	Siegrist et al. (1997)
Tomato	<i>Xanthomonas campestris</i> <i>Alternaria solani</i> <i>Fulvia fulva</i> <i>Oidium</i> sp. <i>Liriomyza</i> spp. <i>Bemisia argentifolii</i>	Peroxidase Lysozyme Chitinase β -1,3-glucanase	Inbar et al. (1998)

Grape	<i>Plasmopara viticola</i>	Eight putative SAR genes	Wong et al. (1998)
Sugar beet	<i>Cercospora</i>	Peroxidase Chitinase β -1,3-glucanase	Braun-Kiewnick et al. (1998a). (Burketová et al. 1999)
Barley	<i>P. syringae</i>	Peroxidase β -1,3-glucanase Chitinase	Braun-Kiewnick et al. (1998b)
Maize	<i>Peronosclerospora sorghi</i>	PR-1 PR-5	Morris et al. (1998)
Soybean	<i>Sclerotinia</i> sp. <i>Clerotiorum</i>	NM	Dann et al. (1998)
Melon	<i>Colletotrichum lagenarium</i> CMV	Chitinase Peroxidase	Smith-Becker et al. (1998)
Rice	<i>Magnaporthe grisea</i>	A set of defence genes	Schweizer (1999)
Cauliflower	<i>Peronospora parasitica</i>	NM	Godard (1999)
Sunflower	<i>Plasmopara helianthi</i>	NM	Tosi et al. (1999)
Japanese pear	<i>G. asiaticum</i> <i>V. nashicola</i>	NM	Ishii et al. (1999)
Cotton	<i>Alternaria macrospora</i>	β -1,3-glucanase	Colson-Hanks and Deverall (2000)

NM = not mentioned.

1.3.2.3 Conditioning

Plants that are pretreated with a necrotising pathogen or a synthetic inducer of SAR, react more rapidly and more efficiently to a challenge infection in the systemically protected leaves. This phenomenon is referred to as conditioning or sensitising (Sticher et al. 1997). The protected tissue not only becomes more resistant and reacts more rapidly to pathogen infection, but also has an altered reaction to environmental stress. Although the conditioning phenomenon has long been known, little is known about the molecular and biochemical mechanism that mediates the conditioning (Sticher et al. 1997).

The biochemical changes occurring in a conditioned plant usually only become apparent at the moment of a challenge infection. There is growing evidence to support this hypothesis. Preincubation with the natural or synthetic SAR inducers such as SA or INA in a time-dependent manner enhances the coumarin secretion and incorporation of phenolics into the cell wall in parsley cell, or enhances formation of lignified papillae in cucumber (Sticher et al. 1997). Mur et al. (1996) showed that SA can potentiate the expression of defence genes that are not activated directly by SA but became induced after pathogen attack or wounding. INA, SA or BTH do not only affect expression of the cucumber chitinase gene as direct inducers, but also act synergistically with fungal elicitors (Kästner et al. 1998). Pretreatment of the parsley cell cultures with SA or BTH leads to the activation of PAL gene in response to low-dose elicitation. In contrast to the PAL genes, SA or BTH directly induces anionic peroxidase in the absence of elicitor. Thus, they probably have a dual role in the activation of defence genes (Katz et al. 1998, Thulke and Conrath 1998). These observations strongly suggest that SA and other SAR inducers can enhance the potency of plant tissue to react better with a variety of complex local defence responses, upon the challenge infection. It has been speculated that hitherto unknown components of the signal perception or transduction pathway might be induced (Sticher et al. 1997).

1.3.3 Roles of PR proteins in disease resistance

The strong correlation between the development of HR or SAR and the accumulation of a set of PR proteins suggests that PR proteins may play an important role in plant defence response. This hypothesis is further supported by the discovery of many PR proteins possessing antimicrobial activities *in vitro* and *in vivo* (see following).

The biochemical function of PR-1 protein is not yet known (Stintzi et al. 1993). However, several PR-1 protein isoforms isolated from tomato and tobacco have been shown to possess antifungal activity *in vitro* (Niderman et al. 1995). In addition, the overexpression of the acidic PR-1a gene in transgenic tobacco plants has demonstrated a significant increase in resistance to infection by two fungal pathogens, suggesting that this gene family or at least some members of it, can play a role in disease resistance (Alexander et al. 1993). Nevertheless, constitutive expression of the PR-1a or PR-1b genes in transgenic tobacco plants did not show higher levels of resistance to virus infection (Stintzi et al. 1993).

β -1,3-glucanases may play a direct role in the plant's defence against fungal pathogens because these hydrolytic enzymes catalyse the degradation of β -1,3-glucan, which is a major cell wall component of many fungal pathogens (Bartnicki-Garcia 1970, Boller 1993). It has been proposed that extracellular β -1,3-glucanases have a defence role during the early fungal pathogen infection process. While the vacuolar class I β -1,3-glucanases may act late in the infection process when cell breakage releases cellular contents into the extracellular compartment (Mauch and Staehelin 1989). β -1,3-glucanases and chitinase may also be involved in the stimulation of the plant defence reactions by releasing oligosaccharides from fungal cell walls and plant cell walls that can induce the accumulation of phytoalexins, extensin, proteinase inhibitors and lignin in host cell (Ryan 1988, Mauch and Staehelin 1989, Takeuchi et al. 1990). Evidence supporting the role of β -1,3-glucanases in the resistance response has been provided from reports demonstrating antifungal effects both *in vitro* and *in vivo* (Mauch et al. 1988, Sela-Buurlage et al. 1993, Zhu et al. 1994, Jongedijk et al. 1995). For example, β -1,3-glucanase isoforms isolated from pea pods inhibited the growth of *Fusarium solani* *in vitro* (Mauch et al. 1988). Transgenic tobacco plants

constitutively expressing the β -1,3-glucanases (PR-N) showed an increased resistance to the pathogens *Phytophthora parasitica* var. *nicotiana*, *P. tabacina* and *Alternaria alternata* (Lusso and Kuc' 1996). In contrast, reducing the expression of the β -1,3-glucanase gene (PR-N) by generating antisense plants resulted in a lower level of resistance (Lusso and Kuc' 1996). Recently, it is of interest to find that tobacco plants deficient in class I β -1,3-glucanases showed a marked reduction of disease severity of tobacco mosaic virus (Beffa et al. 1996).

Chitinases have been thought to be an active component in the plant defence response (Stintzi et al. 1993). Evidence to support the role of plant chitinases can be drawn from the observations that chitinases isolated from different tissues of dicotyledonous and monocotyledonous plants have antifungal activity *in vitro* (Schlumbaum et al. 1986, Verburg and Huynh 1991, Stintzi et al. 1993). For instance, purified chitinases from maize seeds have antifungal activity against the growth of *Trichoderma reesei*, *Alternaria solani*, and *Fusarium oxysporum* (Huynh et al. 1992). Purified *Arabidopsis* chitinase effectively inhibits the growth of *Trichoderma reesei* (Verburg and Huynh 1991). Both basic class I and acidic class II chitinases have been found to have antifungal activity *in vitro*. Thus, it was assumed that the catalytic domain functions alone mediate its antifungal activity. However, the class I enzymes containing a chitin-binding domain have slightly greater antifungal activities (Yun et al. 1996). Some chitinases have lysozyme activity and these enzymes may be required for the defence against bacterial pathogens (Metraux et al. 1989). The significance of plant chitinases in disease defence is further highlighted by many studies showing that constitutive expression of chitinase in transgenic plants can lead to an increased resistance to pathogens (Stintzi et al. 1993, Van Loon 1997). For example, transgenic tobacco plants constitutively expressing a bean vacuolar chitinase gene showed resistance to *Rhizoctonia solani* (Broglie et al. 1991). Expression of rice chitinase in transgenic cucumber and rose plants resulted in their enhanced resistance to grey mold (Tabei et al. 1998) and blackspot (Marchant et al. 1998), respectively.

There is strong evidence that chitinases, especially in combination with β -1,3-glucanases participate in the defence of plants against fungal pathogens (Stintzi et al. 1993, Zhu et al. 1994, Van Loon 1997). β -1,3-glucanase and chitinase are simultaneously induced and associated with resistance in response to various pathogen infection and abiotic agents (Kim and Hwang 1996, Yi and Hwang 1996, Hwang et al. 1997). The vacuolar class I chitinases and class I β -1,3-glucanases have been shown to synergistically inhibit fungal growth *in vitro* (Mauch et al. 1988, Leah et al. 1991, Sela-Buurlage et al. 1993). Furthermore, transgenic tomato plants simultaneously expressing tobacco class I chitinase and class I β -1,3-glucanase gene led to an increase of resistance to *Fusarium oxysporum*, whereas transgenic tomato plants constitutively expressing either one of these genes were not efficiently protected against fungal infection (Jongedijk et al. 1995). In addition, the intracellular class V chitinases and class I β -1,3-glucanases also seem to act in such a synergy (Melchers et al. 1994). However, there are other examples showing that this is not the case (Neuhaus et al. 1991, Neuhaus et al. 1992). A possible explanation for these contradictory results is that not all chitinases or glucanases exhibit antifungal activity (Sela-Buurlage et al. 1993).

The combination of chitinase with other PR proteins also exhibits antifungal activity such as tobacco class I chitinase and class I PR-4 protein (Ponstein et al. 1994a). Purified class I PR-4 protein from tobacco was shown to have antifungal activity against several fungi and to act synergistically with both a tobacco class I chitinase and a class I β -1,3-glucanase *in vitro* (Ponstein et al. 1994b). However, so far, no results have been published on suppression of pathogens in transgenic plants expressing PR-4 gene.

The PR-5 proteins from different plant species have been reported to possess antifungal activity against a variety of fungi *in vitro* through the lysis of spores, inhibition of hyphal growth and/or the reduction of spore germination (Roberts and Selitrennikoff 1990, Vigers et al. 1991, Malehorn et al. 1994, Koiwa et al. 1997). Transgenic plants expressing constitutively PR-5 gene were not protected from infection by TMV (Linthorst et al. 1989), but was found to have enhanced resistance

to fungal pathogen infection (Liu et al. 1994). The mechanism of antifungal action of PR-5 proteins has been proposed to work in two steps involving binding β -1,3-glucans to fungal cell walls and membrane permeabilization (Trudel et al. 1998).

PR-6 proteins are proteinase inhibitors. It has been proposed that they are involved in plant defence against insects and other herbivores, microorganisms, and nematodes (Van Loon and Van Strien 1999).

PR-7 protein was found in tomato as an endoproteinase. It is assumed that it takes part in some antifungal action (Van Loon and Van Strien 1999).

There is increasing evidence that enhanced peroxidase activities are associated with plant disease resistance (Flott et al. 1989, Irving and Kuc' 1990, Dalisay and Kuc 1995a, Chittoor et al. 1997, Xue et al. 1998). Peroxidases are primarily localised in intercellular spaces where they make contact with pathogens, suggesting that they may play an active role in the defence reaction. These enzymes are involved in lignification (Hammerschmidt and Kuc 1982) and cross-linking of extensin (Everdeen et al. 1988). Peroxidases also prevent the accumulation of hydrogen peroxide, which is highly toxic to a living cell (Scandalios 1993). In addition, peroxidases are capable of oxidising phenolic compounds, which may inhibit pathogen development (Retig 1974).

Although many PR proteins have been shown to possess antifungal activity *in vitro* and *in vivo*, so far, none of the characterised proteins probably have direct antiviral and antibacterial activity. Even transgenic plant constitutively expressing PR proteins did not produce virus resistance (Linthorst et al. 1989). However, the accumulation of PR proteins is strongly correlated with plant resistance to viral and bacterial pathogen (Stintzi et al. 1993, Ryals et al. 1996, Van Loon 1997). Basing on the synergistic actions of some of the PR proteins when expressed together, it has been inferred that coordinately expressed multiple SAR genes may lead to yield a strong anti-pathogenic potential. In addition, it was thought that PR-8 family of chitinases, possessing lysozyme activity, might inhibit the growth of bacteria (Van Loon 1997). Recently, Clarke et al. (1998) reported that the expression of PR

proteins might be due to virus-induced endogenous hormone imbalance. Therefore, PR proteins may play an indirect role in defence response to viruses by triggering senescence, cell death and/or cell wall modifications which may restrict virus movement.

1.4 An introduction to roses

1.4.1 Roses

Roses are the most popular garden plants in the world as well as the most important commercial cut flowers. They are cultivated for their beautiful flowers and their fragrances in the perfume industry (Horst 1983).

Roses belong to the *Rosaceae* family. The name rose is derived from its largest genus *Rosa*, which is only found naturally occurring in the northern hemisphere. Species are distributed in four regions: Europe, America, Asia and the Middle East. Roses have been cultivated as early as 2350 BC. They were introduced in New Zealand in the nineteenth century (McKeon 1995).

Roses are classified according to their ancestry into 3 classes: wild roses, old roses, and modern roses. Modern roses have been developed through breeding and selection for more than 130 years. Modern roses are further classified into several distinct groups based on habits of growth, manners of flowering and types of blooms such as hybrid tea, floribunda, miniature, and climbing (Martin and Hutchinson 1992).

The rose is polycarpic self-inductive plant. Its flower is initiated autonomously on every growing shoot after a certain size is attained without a specific photoperiod or temperature requirement (Halevy 1986).

1.4.2 Culture

Roses can be propagated vegetatively, mainly by grafting or using tissue culture methods.

1.4.2.1 Grafting

Traditionally, most quality roses are propagated by bud grafting. However, this method is a very slow, time consuming, and back-breaking process (Skirvin et al. 1990).

1.4.2.2 Tissue culture

Various rose species and hybrids have been cultured *in vitro*. Tissue cultures of roses are established from a variety of explants such as axillary buds, shoot tips, anthers, embryos and calli which may be initiated from leaves, stems, roots and other plant parts (Hasegawa 1980, Skirvin et al. 1990).

Tissue culture is important for commercial propagation of roses. It is well known that tissue culture plants can be variable (Skirvin et al. 1994), but, in most cases, roses have been quite stable, particularly when propagated via axillary buds (Skirvin et al. 1990). Through micropropagation, greater numbers of identical rose plants can be produced in a much short time and eliminated the use of rootstocks for certain cultivars (Skirvin et al. 1990).

Regeneration of shoots from callus is a critical requirement for the successful application of somaclonal variation, genetic engineering techniques and fast propagation. Although rose plant regeneration is far from being routine, recently many regeneration procedures have been developed (Burger et al. 1990, Skirvin et al. 1990, Rosu et al. 1995). For example, adventitious shoots can be obtained from excised leaves, roots and callus of *Rosa × xanthina* (Loyd et al. 1988), stem callus (Ishioka and Tanimoto 1990), proliferating shoots of a chimera *Rosa multiflora* rootstock (Rosu et al. 1995) and immature embryos of *Rosa hybrida* L. (Burger et al. 1990).

Embryogenic cell culture is a promising material for protoplast culture, somatic hybridisation and genetic transformation. Recently, several investigations have demonstrated the induction of somatic embryogenesis on calli derived from rose immature seed (Kunitake et al. 1993), immature leaf and stem segments (Rout et al. 1991), adventitious roots (Van der Salm et al. 1996), and stamen filaments of young

flower buds (Noriega and Söndahl 1991). However, the frequency of regeneration of shoots via somatic embryogenesis is still very low in rose.

1.4.3 Rose diseases

Roses growing in the field or garden are subject to numerous diseases. The most important rose diseases are caused by fungi, such as powdery mildew, blackspot and rust. These fungi often cause considerable damages to commercial rose plants and those in home gardens. They are also common diseases of roses in New Zealand. Several other fungal pathogens also affect rose growth, but are of less economic importance. In addition, many bacteria, viruses and nematodes could also cause rose diseases (Horst 1983, Coyier 1985).

1.4.3.1 Fungal diseases

Powdery mildew is caused by *Sphaerotheca pannosa*. This disease occurs worldwide and could seriously influence rose production, particularly cut flower rose plants in glasshouse. The development of the disease depends on environmental conditions and the susceptibility of host plants to pathogen infection. The symptoms first appear as small and discrete lesions on the stems, leaves, and even on the flower parts. As the disease develops, the infected parts are covered by white powdery consisting of mycelium and conidiophores (Coyier 1985, Bélanger and Labbé 1994, Gullino and Garibaldi 1995).

Blackspot, caused by *Diplocarpon rosae*, is a widespread and devastating disease of nursery- and garden-grown roses. Symptoms of this disease can be seen as irregularly-shaped black lesions whose margins are usually fringed with a feathery hyphal growth and often surrounded by chlorosis, on the adaxial surface of leaflets. Severe infection can result in defoliation and dieback of plants. Thus, it reduces plant vigour, the number of flowers, the life of plant and even its marketable value (Coyier 1985, Walker and Mandegaran 1995, Xue and Davidson 1998).

Rose rust is also a widespread and serious disease caused by nine species of the rust fungus. The disease first appears on leaves and other green parts of the plant as

powdery pustules of orange aeciospores on the lower leaf surface. As pustules develop, they become visible on upper leaf surfaces as orange or brown spots. Rust infection can cause defoliation of rose plants (Horst 1983, Coyier 1985).

1.4.3.2 Bacterial diseases

Crown gall, caused by *Agrobacterium tumefaciens*, is an extremely common disease in greenhouse and outdoors-grown roses. With the application of new propagation and modern growing techniques, crown gall has recently become more widespread. Plants with galls grow poorly and are made unsaleable (Horst 1983, Coyier 1985, Gullino and Garibaldi 1995).

1.4.3.3 Viral diseases

Since rose propagation mainly depends on grafting or using tissue culture methods, virus infection has been a permanent problem in rose production. Rose mosaic is a disease caused by a complex of plant viruses, the most well known being prunus necrotic ringspot virus (PNRV). It is spread by the use of infected bud and rootstock. Since PNRV is not fatal to plants, there is little concern by nurseryman and rose grower. However, it reduces plant vigour, flower size and number, delays the onset of flowering, and increases the production of deformed flowers, which directly affects the sales of cut flowers (Thomas 1982, Horst 1983, Coyier 1985). PNRV is widespread in rose plants in New Zealand, therefore, roses from this country have a relative low export potential (Gardner 1983).

1.4.4 Disease control

1.4.4.1 Sanitation and disposal of infected tissues

Sanitation is important in control some rose diseases such as crown gall and stem and graft canker. The incidence and severity of some rose diseases can be significantly reduced by the application of good horticultural practices. Some diseases may be controlled by immediate disposal of infected plants (Coyier 1985, Gullino and Garibaldi 1995).

1.4.4.2 Application of chemicals

Numerous fungicides have been used to control rose fungal pathogens such as powdery mildew and blackspot (Horst et al. 1992, Bélanger and Labbé 1994, Gullino and Garibaldi 1995). However, some negative effects have arisen from the use of fungicides. Firstly, the use of chemicals raises production costs. For example, fungicides are sprayed every 2 weeks for 7 months of a year to control rose diseases caused by fungi in South Pacific Rose Nurseries Ltd of New Zealand. According the market value of cut rose plants of the United States in 1987, the annual cost of fungicides for control of powdery mildew on production roses was 2% of the wholesale market value (Horst et al. 1992). Secondly, many fungicides are moderately effective. Some of fungicides often cause phytotoxicity or result in unacceptable visible residue on the foliage and blossoms, which reduce the commercial value of cut rose flowers (Coyier 1985). Meanwhile, use of fungicides may increase the risk of fungicide resistance being developed. In addition, there is increased recognition that the use of chemicals in the control of plant diseases will become restricted as they may have harmful side effects on the environment and human health (Herrera-Estrella et al. 1996). Therefore, the development of new strategies based on a plant's own defence mechanisms for disease control is critical for sustaining agricultural production and improving our environment and health (Yang et al. 1997).

1.4.4.3 Biological control

Biological control of plant diseases is an effective method for reducing damage from diseases. However, this method is not well applied in rose growing nurseries (Gullino and Garibaldi 1995). For example, *Agrobacterium radiobacter* strain K-84 is used as a biocontrol agent against *A. tumefaciens*, but not always successful on roses. The yeastlike fungus *Sporothrix flocculosa* was reported to control rose powdery mildew, but its efficacy varied with the levels of relative humidity (RH) (Bélanger and Labbé 1994). This is because biocontrol agents must be carefully selected and handled to assure survival of the beneficial organisms (Coyier 1985). Moreover, they are usually effective within a limited range of temperature and RH (Blakeman and Fokkema

1982). In addition, the methods of biological control for most rose diseases are not well investigated. Probably due to these limitations, it seems that a few biocontrol agents have good prospects for successful use on roses (Gullino and Garibaldi 1995).

1.4.4.4 Improved resistance by breeding

One of the means to control diseases is the selection of disease resistant cultivars. However, the availability of rose cultivars with multiple disease resistance is limited. Very few rose species and cultivars are resistant to some pathogens such as blackspot caused by *Diplocarpon rosae* (Svejda and Bolton 1980). It is also not easy to select powdery mildew resistant varieties because several races of the pathogen exist (Gullino and Garibaldi 1995). In addition, relatively little work has been done on resistance breeding in this crop as research has been mainly focused on improving other horticultural traits such as productivity and flower colour. Another reason for limited rose resistance breeding is the difficulty in the production of disease-resistant varieties without disruption or loss of existing desirable characteristics (Preece and Merkle 1997).

1.5 New approaches to improve disease resistance

1.5.1 Somaclonal variation

The regeneration of plants via a callus can frequently lead to plants that are phenotypically and genotypically different from their progenitor. This is referred to as somaclonal variation (Harms 1992, Hutchinson et al. 1992). Somaclonal variation comes from both pre-existing genetic variation within the explants and variation generated by the tissue culture procedures with rates of 15% to 20% (Skirvin et al. 1994). It has been used to select disease resistance *in vitro* or at the plant level, especially under a selective pressure that can be provided by pathogens, culture filtrates of pathogens, or isolated pathotoxins. The selection will allow only those cells that survive and proliferate under pressure (Van den Bulk 1991). Many disease-resistant plants are obtained through somaclonal variation and selection, such as potato plants with resistance to *Verticillium dahliae* (Sebastiani et al. 1994), and apple

rootstocks with resistance to *Phytophthora cactorum* (Rosati et al. 1990). In rose, somaclonal variation has been studied (Carole Pellegrino and Gudin 1993) and some somaclones from leaf explant-derived calli of *Rosa hybrida* demonstrated moderate resistance to powdery mildew (Chatani et al. 1996).

1.5.2 Genetic engineering of plants

Genetic engineering is the most powerful approach for crop improvement (Harms 1992). In order to be efficient in plant transformation, it is necessary to have a good regeneration system to produce transgenic plants, an efficient system for foreign gene transfer, and a suitable gene which confers a desired trait (Hutchinson et al. 1992). The technique of foreign gene transfer to plant is being applied to genetically modify floricultural crops. The most widely used techniques are either *Agrobacterium*-mediated transformation or direct gene transfer by particle bombardment (Robinson and Firoozabady 1993).

Several defence-related proteins have been isolated and cloned. A number of plant genes conferring resistance to pathogens have been identified (Fraser 1990, Hammond-Kosack and Jones 1996). Transgenic plants expressing PR protein genes with enhanced disease resistance have been demonstrated in several crops (Broglie et al. 1991, Liu et al. 1994, Zhu et al. 1994). For example, transgenic cucumber harbouring a rice chitinase gene (RCC2) exhibited enhanced resistance against gray mold. The transgene was confirmed to be inheritable. It is considered as a new breeding material because naturally occurring resistance materials have not been found in the existing genetic resources of cucumber (Tabei et al. 1998). It was also demonstrated that transgenic rose plants expressing a rice chitinase gene led to an increased resistance to *D. rosae* (Marchant et al. 1998). More effective disease resistance in rose plants may be achieved by expressing a rose defence gene. Thus, transgenic plants encoding PR genes may enrich genetic resources for disease resistance in plants including roses. In addition, genetically engineered ornamental crops may be more easily accepted by consumers than genetically engineered agricultural crops as ornamentals are not grown as food crops.

1.5.3 Activation of plant defence mechanisms: BTH application

A new alternative way for protecting crops from diseases has arisen in the last few years. This is to activate plant's own defence mechanism by either biotic or abiotic plant defence elicitors (Kessmann et al. 1994, Ryals et al. 1996). Induced resistance has been demonstrated in at least 20 plant species from at least six different plant families and is likely to be a ubiquitous higher plant defence response (Uknes et al. 1996, Sticher et al. 1997, Lucas 1999). Induced resistance is effective against a broad spectrum of pathogens. The time needed for the establishment of induced resistance varies from hours to weeks and depends on both the plant and the type of inducing elicitors. The protection often lasts weeks or even months. The degree of disease control obtainable through induced resistance is high, with values from 50-90% reported in the literature (Van Huijsduijnen et al. 1986, Lucas 1999). Therefore, it is competitive with the use of resistant cultivars or fungicides.

Chemicals that activate plant's own defence mechanism in the field will provide a promising alternative approach to control plant disease, which is effective and economical and would reduce the dependence on more hazardous agrochemicals for crop protection (Kessmann et al. 1994, Lucas 1999). Among the chemical activators of disease resistance, SA, INA and BTH are intensively studied and well characterised (see chemical induction of SAR). Both SA and INA are capable of inducing resistance against pathogen attack in several crops, but they can readily cause phytotoxic side effect in some crop plants, thus limiting their practical use as plant protection agents. However, most plants tolerate BTH and are protected efficiently by this chemical. It has been thought that BTH is the most potent inducer of both resistance and gene expression and has great promise as a disease control agent in field plants (Görlach et al. 1996). Recently, BTH is introduced in the market as a commercial product (trade name Bion 50WG). In Europe, BTH has been used successfully to protect wheat plants against powdery mildew in the field (Görlach et al. 1996).

Although BTH has been described to induce the resistance against a wide range of pathogens in a wide variety of plant species including dicotyledonous and

monocotyledonous plants, in the greenhouse as well as under field conditions (Table 1.1), potential application of BTH to ornamental plants for disease control has not been investigated yet. Rose is one of the most important flowers in the world, but little information is available about its natural disease defence systems. Up to now, there has been only one report concerning the induced resistance by INA in rose (Hijwegen et al. 1996). Therefore, it is tempting to investigate the ability of BTH to induce rose plant's resistance against diseases.

1.6 The objectives of this study

There is a gap in our knowledge about rose disease defence mechanism and occurrence of PR proteins. The aim of this study is to investigate the natural defence capabilities against pathogens in rose plants, especially the occurrence, characteristics and regulation of PR proteins, and their relationship to disease resistance mechanism. In order to investigate the natural defence mechanism used by rose plants against pathogen attack, BTH, a recently developed novel synthetic compound, is used to stimulate rose plant's defence mechanisms as BTH has already been demonstrated to induce broad-spectrum and long-lasting disease resistance in several plant species (Friedrich et al. 1996, Görlach et al. 1996, Lawton et al. 1996). Research on BTH is of particular interest because it will not only increase our knowledge on the nature of plant defence response, but may also lead to an alternative approach for the disease control in rose. Once induced resistance has been demonstrated in rose, the molecular or biochemical mechanisms behind the phenomenon would need to be studied. Thus far in many plant species studied, BTH-induced resistance is tightly associated with increased accumulation of many classes of PR proteins, most of which are acidic proteins that are secreted into the intercellular space of the leaf (Friedrich et al. 1996, Lawton et al. 1996, Siegrist et al. 1997). Thus, it would be interesting to investigate the protein changes in the intercellular fluids from rose leaves after BTH treatment to understand the disease resistance mechanisms. The present plan of investigation has included the induction patterns of extracellular proteins in response to various concentrations of BTH and the kinetics of induced extracellular protein accumulation in relation to development of disease resistance through the analysis of SDS-PAGE

and 2D-PAGE. To further assist a study of the changes in gene expression specifically induced by BTH, an antiserum against the intercellular fluid of BTH-treated rose leaves is required. Through differential immunoadsorption techniques, the antibodies against BTH-induced antigens could be enriched.

In both *Arabidopsis* and tobacco, two model plants for studying SAR, the same set of PR genes is coordinately induced by BTH treatment and pathogen infection (Ward et al. 1991b, Uknes et al. 1993, Friedrich et al. 1996, Lawton et al. 1996). Therefore, it would be also interesting to know whether the corresponding genes are also induced by BTH treatment and pathogen attack in rose. However, little information is available regarding the occurrence of PR proteins after pathogen infection in rose. Thus, *D. rosae*, which causes local lesions as black leaf spots in rose plants (Wiggers et al. 1997), is used to inoculate rose shoots and investigate the expression and accumulation of extracellular PR protein in response to the fungal infection. Meanwhile, the intercellular fluids extracted from the pathogen-infected or BTH-treated leaves of rose shoots are to be examined by the immunoblot technique for the induction and accumulation of four major classes of PR proteins. This was aided with several appropriate tobacco antibodies kindly made available by Dr Kauffmann (Institute of Plant Molecular Biology, CNRS, Strasbourg, France).

Chitinase, β -1,3-glucanase and peroxidase have been extensively studied due to their involvement in the disease resistance response of many crops (Goy et al. 1992, Dalisay and Kuc 1995a, Yi and Hwang 1996, Xue et al. 1998). The aim of this study is also to test if BTH induces chitinase, β -1,3-glucanase and/or peroxidase in the intercellular spaces of leaves and if the activities of these enzymes are correlated with induced resistance in rose. From this, the activities of these enzymes in BTH-pretreated and water-pretreated leaves with subsequent challenge inoculation with *D. rosae* are to be determined and further characterised by analysis of patterns of their acidic and basic isoforms.

The final objective of this study is to ascertain if the BTH-induced extracellular proteins, which are associated with disease resistance, are also induced by SA,

ethephon, or HgCl_2 . The possibility that they are expressed in normal pot plants or senescent leaves *in vitro* will also be examined.

In summary, the objectives of this study are:

- ◆ To test whether a BTH pretreatment can induce disease resistance in rose shoots to subsequent challenge inoculation with *D. rosae* (blackspot causal agent) or *A. tumefaciens* (crown gall causal agent).
- ◆ To investigate gene expression patterns that are correlated with BTH-induced resistance, focusing on the induction and characterisation of extracellular PR proteins.
- ◆ To explore the possibility that the BTH- or *D. rosae*-induced extracellular proteins of rose are serologically related to tobacco PR-1, PR-2, PR-3, and PR-5 proteins, respectively.
- ◆ To determine if the activities and specific isoforms of extracellular β -1,3-glucanase, chitinase and peroxidase are induced by BTH, or *D. rosae* and if they might be related to disease resistance in rose plants.
- ◆ To study the induction of the four major classes of extracellular PR proteins by a range of treatments to ascertain whether BTH specifically induced the proteins.

Chapter 2

MATERIALS AND METHODS

2.1 Sources of chemicals

Ascorbic acid, β -alanine, bovine serum albumin, bromphenol blue, 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt, chitinase, Coomassie Brilliant Blue R-250, o-dianisidine, fluorescent brightener 28, glycine, guaiacol, laminarin, p-nitrophenyl α -D-mannopyranoside, neocuproine, nitro blue tetrazolium, Pharmalyte 2D pH 3-10, polyvinylpolypyrrolidone, 2,3,5-triphenyl-tetrazolium chloride, Tris and urea were obtained from Sigma, St. Louis, MO, USA.

2-D SDS-PAGE standards, ammonium persulfate, IEF Standards Broad Range pI 4.45-9.6, piperazine di-acrylamide, polyvinylidene difluoride, prestained SDS-PAGE standards (low-range), SDS-PAGE molecular weight standards (low range), TEMED and thioglycolate were from *BIO-RAD*, Hercules, CA, USA.

Acrylamide, N,N'-methylene bisacrylamide, citric acid, formaldehyde, glacial acetic acid, hydrogen peroxide, β -mercaptoethanol, methylene-blue, Resolyte pH 4-8, silver nitrate, sodium dodecyl sulphate and trichloroacetic acid were purchased from BDH chemicals Ltd, Pool, England.

Anti-rabbit IgG-AP (immunoglobulin) was obtained from Boehringer Mannheim, GmbH-Germany. CM-Chitin-RBV was from Loewe Biochemica, Sauerlach, Germany.

BTH (trade name CGA 245704 Bion 50WG) as in a water dispersible granule form with 50% active ingredients was kindly supplied by Novartis Crop Protection Australasia Limited.

Potato dextrose agar was from GIBCOBRL, LIFE TECHNOLOGIES, Paisley, Scotland.

2.2 Plant materials

2.2.1 *Rose varieties*

Alexander (hybrid tea), the brightest of the orange-vermilion roses, was introduced to New Zealand in 1972. It was from Super Star × Anne Elizabeth × Allgold, and can grow to 1.5-1.8 meters and is exceptionally healthy and disease free (Martin and Hutchinson 1992).

Iris Gee (hybrid tea-floribunda) is a tall garden rose plant with dainty creamy peach blooms.

Madame Isaac Pereire (Bourbon) is one of the most fragrant roses. It is a tall and bushy plant with large deep pink flowers, and is susceptible to blackspot (McKeon 1995)

2.2.2 *Culture of rose shoots in vitro*

Rose plants, obtained from Pacific Roses Orchard (Christchurch, New Zealand), were propagated *in vitro* as follows. After removal of leaves, nodal sections about 1 cm long (each with an axillary bud) were excised and surface sterilised by immersion in 70% (v/v) ethanol for 30 second and 2.4% (w/v) sodium hypochlorite (NaOCl) for 20 min, and followed by three rinses in sterile distilled water.

Each explant was cultured in a culture jar (\varnothing 4.2 × 5.5 cm) containing 15 ml of MS (Murashige and Skoog 1962) medium, supplemented with 1 mg/l

benzylaminopurine (BA), 40 g/l sucrose and 8 g/l agar. Three weeks after bud break, new shoots were removed from the stem tissue and transferred to a shoot multiplication medium, which was MS medium supplemented with 40 g/l sucrose, 2 mg/l BA, 0.1 mg/l gibberellic acid (GA₃), 0.004 mg/l naphthalene acetic acid (NAA) as described by Davies (1980). The pH of all media was adjusted to 5.7 with NaOH before the addition of agar. Media were autoclaved at 121° C for 20 min. For continuous *in vitro* propagation, the shoots about 1 cm long were subcultured every 4 weeks on the shoot multiplication medium.

Shoots about 3 cm long were subcultured on MS medium, in which roots were formed after 2–3 weeks.

The cultures were grown at 22°C under 24 h photoperiod with a light intensity of 50 $\mu\text{E m}^{-2} \text{s}^{-1}$ provided by warm white fluorescent tubes.

2.2.3 Growth of tissue culture-derived rose plants in soil

Rooted shoots grown *in vitro* were washed with tap water to remove agar before they were transplanted into individual plastic pots (6 × 6 × 8 cm). The potting mix comprised sterilised standard potting compost (63% tree bark, 20% peat, 8.5% soil, 8.5% sand and 270-day Nutricote, three-month Nitrophoska blue, potassium sulphate, calcium ammonium nitrate and trace elements). To acclimatize plantlets to *ex-vitro* conditions, each pot was first covered with a plastic bag for one week and then gradually lowered the humidity to a normal condition of growth chamber within one more week. Afterwards, the plants were grown in a growth chamber at 21°C with 16 h photoperiod, and were watered 2 times every week.

The major stages of the rose tissue culture system here are summarised in Plate 2.1.

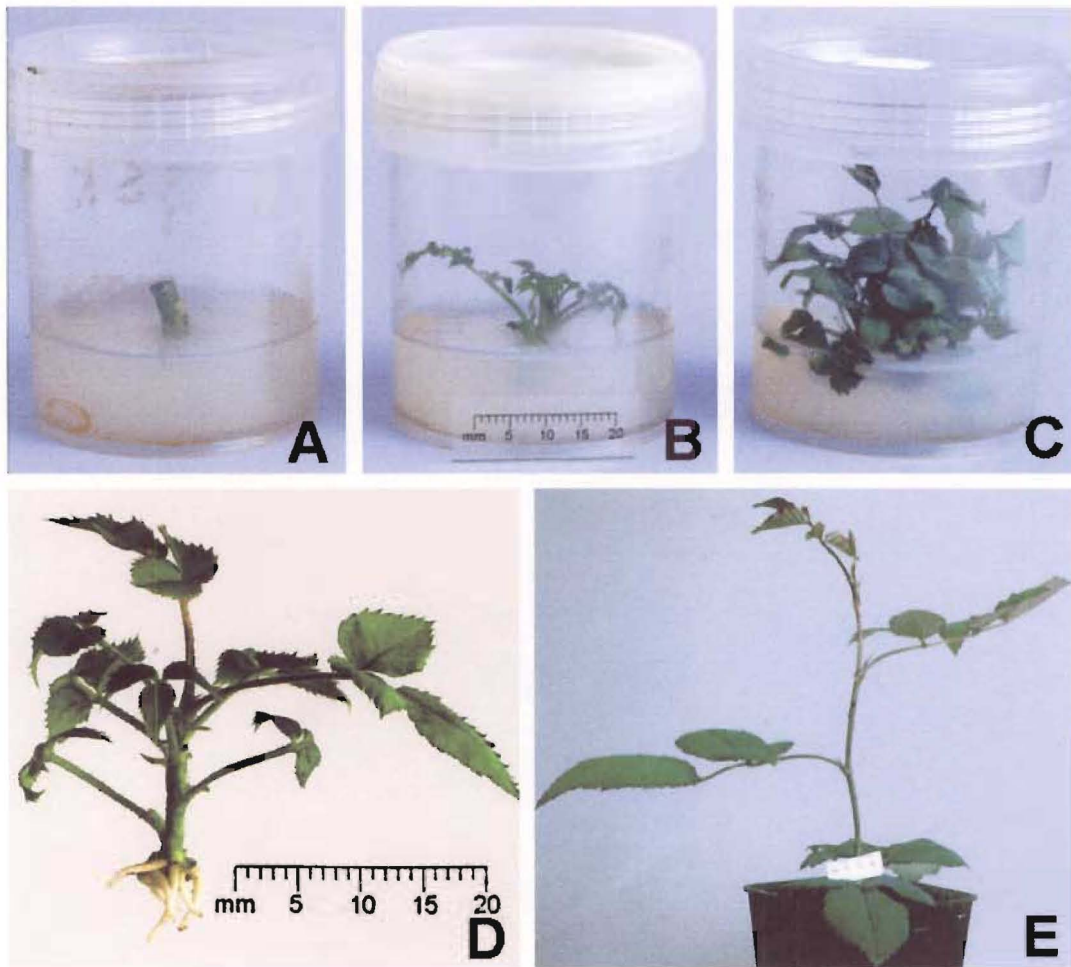


Plate 2.1 The rose tissue culture system in this study.

(A) nodal section with an axillary bud; (B) new single shoot excised and subcultured after bud break; (C) proliferation of shoots after subculture; (D) rooting of a 3 cm *in vitro* shoots 2-3 weeks after subculture; (E) a tissue culture-derived plant established successfully under *ex-vitro* conditions. The photographs showed material of the rose var. Iris Gee.

2.3 Chemical treatments

Shoots about 3 cm long were first subcultured on MS medium without any plant growth regulator. Five days later, these shoots were used for chemical treatments. For each treatment, approximately 30 shoots placed in 5 jars each with 6 shoots were randomly chosen. At the time of treatment, the shoots had about 4 to 6 leaves.

BTH (trade name Bion 50WG), in a water dispersible granule form containing 50% active ingredient, was dissolved in water and filter sterilised or autoclaved. The whole rose shoots were either dipped in different concentrations of BTH solutions for a few seconds to wet all leaves, or in sterile distilled water as a control. After dipping, the shoots were put back to the MS medium. In some experiments, BTH was added directly into MS medium.

Salicylic acid was first dissolved in a small volume of 1 N NaOH, then diluted by distilled water and adjusted to pH 6.5 with 1N HCl. Salicylic acid and ethephon (2-chloroethylphosphonic acid) solutions were filter sterilised. The treatments of rose shoots with salicylic acid, ethephon and mercuric chloride were the same as those with BTH.

2.4 Pathogens and their culture

Diplocarpon rosae was isolated from blackspot infected leaves of rose plants grown in Pacific Roses Orchard according to the published methods (Bolton and Svejda 1979, Xue and Davidson 1998). Leaf discs with the typical blackspot lesions were cut, surface sterilised by immersion in 70% (v/v) ethanol for 30 seconds and 1% (w/v) sodium hypochlorite for 10 min, rinsed three times in sterile distilled water, and then cultured in 9 cm Petri dishes containing potato dextrose agar (PDA) for 2 weeks at 21 °C with 16 h photoperiod. The spores were obtained from the colonies of *D. rosae* that developed on leaf disks and placed into an Eppendorf tube containing sterile distilled water. The resultant conidial suspension was then spread on PDA in a Petri dish and cultured as described above for 24 h. Germinated spores were individually transferred to fresh PDA plates and incubated in the same conditions for one month.

The pathogenicity of the cultures was checked by reinfecting the healthy leaves of rose plants.

A wild-type strain (C58) of *Agrobacterium tumefaciens* streaked from storage was cultured on LB medium developed by Luria and Burrows (1957) in a Petri dish and grown for 2 days at 26°C in the dark. Single colony incubation was made in a 125 ml flask containing 50 ml of LB liquid medium at 26°C with continuous shaking until the optical density of the culture at 600 nm was about 0.6. The culture of C58 with the recombinant plasmid pIG 121 (Ohta et al. 1990), containing a kanamycin-resistance gene (*npt II*) and a β -glucuronidase gene (GUS), was handled in the same way as for C58 except that LB medium was supplemented with 50 μ g/ml kanamycin sulfate.

2.5 Inoculation

2.5.1 *Diplocarpon rosae*

The conidial suspension of *D. rosae* was prepared from about one month old culture by washing and scraping the surface of colonies with sterile distilled water and a scalpel, then filtered through two layers of cheesecloth to remove mycelial fragments. The number of conidia in the suspension was adjusted to 10^5 /ml following counting with a haemocytometer. Four days after treatment with BTH or distilled water, the rose shoots grown *in vitro* were first washed with distilled water, and then inoculated by dipping them into the conidial suspension of *D. rosae* under otherwise aseptic conditions. After challenge inoculation, the inoculated shoots were immediately transferred to jars (\varnothing 6.5 \times 8.5 cm) containing 40 ml of 0.8% Agar (4 shoots per jar) and were kept in the dark at 21°C for 24 h before returning to normal growth conditions for disease development.

2.5.2 *Agrobacterium tumefaciens*

Four days after treatment with BTH or distilled water, the stems of rose shoots grown *in vitro* were inoculated by being stabbed with a previously sterile needle carrying a droplet of the bacterial culture for 2 times at about 1 cm apart. The control shoots were stabbed and inoculated with a droplet of sterile distilled water or LB liquid

medium. Also included for comparison were shoots that were not wounded, i.e. the non-inoculated control. After inoculation, the shoots were put back to culture medium (MS). Each treatment consisted of 24 rose shoots.

2.6 Evaluation of diseases

2.6.1 Determination of conidia germination

Three days after inoculation, the germination of *D. rosae* conidia was examined by epifluorescence light microscope according to the method of Wiggers et al. (1997). Three rose shoots per treatment were selected and immersed in 0.1 M Tris-HCl (pH 8.5) containing 0.1% (w/v) fluorescent brightener 28 for 1 min. After staining, the leaves were washed with water and mounted on glass slides. The number of germinated or ungerminated conidia out of 300 conidia in each treatment was counted.

2.6.2 Histochemical and microscopic observations

2.6.2.1 Blackspot

The development of blackspot on leaves of *in vitro* rose shoots was observed under stereo- microscope at day 1, 3, 5, and 7 after inoculation with *D. rosae*. At the same time, six shoots from each treatment were randomly selected and stained with alcoholic lactophenol-trypan blue as described by Keogh et al. (1980). Their leaves were boiled in alcoholic lactophenol-trypan blue (10 g phenol, 10 ml lactic acid, 10 ml glycerine, 0.01 g trypan blue, 10 ml water, 80 ml ethanol) for 2 min, rinsed with water, decolourised in chloral hydrate (2.5 g /ml) for 2 days, and stored in 50% (v/v) glycerine.

2.6.2.2 Putatively gall tissue

GUS enzyme activity in the putative gall tissue formed following *A. tumefaciens* inoculation was assessed histochemically as described by Jefferson (Jefferson 1987). Galls were excised from the stems and immersed in reaction buffer containing 50 mM sodium phosphate (pH 7.0), 4% methanol and 0.5 mg/ml 5-bromo-4-chloro-3-indoly

glucuronide (X-Gluc). Tissue was stained for 4 h at 37°C and overnight at room temperature before destaining through several changes of 70% ethanol.

2.6.3 Assessment of disease symptoms

2.6.3.1 Blackspot

After 7 days from inoculation with *D. rosae*, the percentage of infected leaves was determined from 24 shoots per treatment in each experiment. The disease severity was estimated by recording leaf area with symptoms (LAS) on 8-point scale according to Xue and Davidson (1998). The eight categories were 0 = no disease [median value (m.v.) = 0%], 1 = blackspots on less than 1% of the leaves (m.v. = 0.5%), 2 = blackspots on approximately 1% to 5% of the leaves (m.v. = 2.5%), 3 = blackspots on 6% to 10% of the leaves (m.v. = 7.5%), 4 = blackspots on 11% to 25% of the leaves (m.v. = 17.5%), 5 = blackspots on 26% to 50% of the leaves (m.v. = 37.5%), 6 = blackspots on 51% to 75% of the leaf (m.v. = 62.5%), 7 = blackspots on more than 75% of the leaves (m.v. = 87.5%). The mean LAS value of leaves per shoot was calculated using the formula below.

$$\text{LAS} = \sum (\text{m.v.} \times \text{No. leaves in a category}) / \text{total No. leaves}$$

2.6.3.2 Crown gall

The frequency of gall or callus formation on *in vitro* rose shoots was scored after 2 or 4 weeks from inoculation with *A. tumefaciens*. The size of the galls/calli was also determined at the indicated times. According the investigation of Cervera et al. (1998), each inoculated site was considered to be an independent replicate.

Each experiment was repeated twice. Data presented in this thesis were averages from two experiments.

2.7 *In vitro* antimicrobial assay

2.7.1 BTH

2.7.1.1 Bacterial growth inhibition assay

The culture of *A. tumefaciens* was grown in LB broth at 26°C with shaking to an optical density of 0.02 at 600 nm. A 50 µl aliquot of this liquid culture was spread onto plastic Petri dishes containing solid LB medium as control or LB supplemented with 50 µM or 0.5 mM BTH, using a sterile plate spreader. The Petri dishes were incubated for 2 days at 26°C in the dark.

2.7.1.2 Fungal growth inhibition assay

Microtitre plate (24 wells) assay as described by (Leah et al. 1991, Woloshuk et al. 1991, Lawrence et al. 1996) was used to determine the effect of BTH on the growth of *D. rosae*. Each well was pipetted with 250 µl potato dextrose broth. BTH, after being filter-sterilised or autoclaved, was also added to each well at a final concentration of either 50 µM or 0.5 mM. To the control, sterile water was added. Five hundred spores of *D. rosae* suspended in 50 µl of sterile distilled water were then added to each well. The microtiter dishes were incubated for 6 days at 21°C in the dark. Mycelia were stained with lactophenol cotton blue (10 g phenol, 10 ml glycerine, 10 ml lactic acid, 0.02 g aniline blue and 10 ml distilled water) and observed under light microscope.

2.7.2 Intercellular fluid (ICF) from BTH-treated leaves

To assay the *in vitro* antifungal effect of intercellular fluid (for preparation, see 2.8.2) on the growth of *D. rosae*, the procedure was the same as described above except that protein solution was added into each well rather than BTH. Protein solutions were boiled for 10 min as controls.

2.8 Protein extraction

2.8.1 Tissue collection

Leaves infected with blackspot were harvested at day 1, 3, 5, and 7 days after inoculation. For the control without inoculation, the leaves were harvested before inoculation and also at the same times indicated for harvesting the inoculated leaves. The newly expanded and upper uninfected leaves were also collected from uninoculated control and 7 days after inoculation with *D. rosae*.

For chemical treatments, leaves were harvested 5 days after treatment.

2.8.2 Inter cellular fluid extraction

The intercellular fluid of rose leaves was collected according to De Wit and Spikman (1982) with slight modification. Entire leaves, harvested at different time intervals after treatment, were washed with distilled water three times and submerged in three different solutions, i.e. 0.1 mM citrate-phosphate buffer pH 2.8; 0.1 M Tris-HCl buffer (pH 8.0) containing 2 mM CaCl_2 , 50 mM 2-mercaptoethanol, 10 mM EDTA, or distilled water in jars (lids off) under vacuum for 30 min. Infiltration was finished when leaves showed a glossy dark green appearance. The leaves were gently blotted dry using paper towels, and transferred into a 2.5 ml plastic syringe barrel. The syringe barrel was then put in a 30 ml centrifugation tube containing a 1.5 ml Eppendorf tube (without lid). In this case, the syringe barrel just sat on the top of Eppendorf tube. After centrifugation at $3000 \times g$ for 10 min at 4°C , the intercellular fluid was recovered in the Eppendorf tube. It was then used immediately or stored at -80°C .

2.8.3 Cell protein extraction

After collection of ICF, the infiltrated leaves were frozen in liquid nitrogen and homogenised to a fine powder with a mortar and pestle. Four volumes of cold 0.1 M sodium acetate buffer (pH 5.2) containing 0.1% (v/v) 2-mercaptoethanol, 10 mM EDTA, 6 mM L-ascorbic acid and 3% (w/v) insoluble PVP were added to the frozen powder, which was then ground until the tissue was thoroughly homogenised. The

crude extracts were centrifuged twice to remove insoluble material at $12,500 \times g$ for 30 min and at $23,000 \times g$ for 30 min at 4°C . After centrifugation, the supernatants were collected and used immediately or stored at -80°C until required.

2.8.4 Protein determination

Protein concentrations of samples were determined for all experiments using the method described by Bradford (1976). Bovine serum albumin (BSA) was used as a standard.

2.9 Gel electrophoresis

2.9.1 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Electrophoresis on denaturing polyacrylamide gels was performed on slab gels following the method of Laemmli (1970), using a 15% separating gel (0.375 M Tris-HCl, pH 8.8, 0.1% SDS) and a 4% stacking gel (0.125M Tris-HCl, pH 6.8, 0.1% SDS). Gels were polymerised with 10% ammonium persulphate as catalyst and TEMED as initiator. Samples were diluted at 3:1 (v/v) in sample buffer (0.15 M Tris-HCl, pH 6.8, 20% glycerol, 2% SDS and 0.05% 2-mercaptoethanol) and boiled for 5 min, then spun at $11000 \times g$ for 5 min before electrophoresis. Gels were run using Tris-glycine buffer (pH 8.4) in a Mini-PROTEAN II electrophoresis cell (*BIO-RAD*) at 100 V constant voltage for 5 min and then 160 V for about 1 h. Some gels were also run in a standard protein electrophoresis unit ($110 \times 150 \times 1.5$ mm for separating gel and $30 \times 150 \times 1.5$ mm for stacking gel) at 60 mA until the dye front had reached the separating gels, and then changed to constant 30 mA for 4.5 h at room temperature.

For silver staining, PDA was used as a crosslinking agent instead of methylene-bis-acrylamide (bis) in polyacrylamide gels to reduce the background staining.

SDS-PAGE Molecular Weight Standards-low range (*BIO-RAD*) was used: phosphorylase b 97.4 kDa, bovine serum albumin 66.2 kDa, ovalbumin 45 kDa,

carbonic anhydrase 31 kDa, soybean trypsin inhibitor 21.5 kDa, and lysozyme 14.4 kDa.

2.9.2 Polyacrylamide gel electrophoresis of acidic proteins

Acidic proteins of ICF extracts were analysed by discontinuous polyacrylamide gel electrophoresis at 4°C under non-denaturing conditions according to Laemmli (1970) with the omission of SDS and 2-mercaptoethanol from all buffers. PAGE was performed on mini slab gels (0.75 mm) with a 10% or 15% separating gel and a 4% stacking gel. Gels were prepared the day before use and stored at 4°C overnight. Running buffer (Tris-glycine, pH 8.4) was prechilled. Each lane was loaded with 10 µg proteins and 0.05% (w/v) bromphenol blue in the sample buffer (see appendix 2) as the tracking marker. Gels were run at a constant voltage of 160 V at 4°C to minimise the possibility of enzyme inactivation.

2.9.3 Polyacrylamide gel electrophoresis of basic proteins

Basic proteins of ICF extracts were analysed by discontinuous polyacrylamide gel electrophoresis at 4°C under non-denaturing conditions as described previously by Reisfeld et al. (1962). PAGE was performed on mini slab gels (0.75 mm) with a 10% separating gel (0.06 M KOH and 0.376 M acetic acid, pH 4.3) and a 4% stacking gel (0.06 M KOH and 0.063 M acetic acid, pH 6.8). Gels were precooled to 4°C before use and maintained at this temperature during electrophoresis. Electrophoresis buffer at pH 4.5 consisted of 0.14 M β-alanine and 0.35 M acetic acid. Samples were mixed at 2:1 (v/v) with 60% (w/v) sucrose containing 0.6% (w/v) methylene blue. Each lane was loaded with 10 µg proteins and methylene blue as the tracking marker. Gels were run at 160 V constant voltage at 4°C until the blue dye reached the bottom.

2.9.4 Isoelectric focusing (IEF)

Isoelectric focusing of ICF extracts was performed according to the method of Bollag et al. (1996b) on a Mini-PROTEAN II electrophoresis cell. The gels (0.75 mm thick) contained 5% acrylamide cross-lined with piperazine di-acrylamide (at the ratio of 30:1), 2.4% (v/v) Pharmalyte pH 3-10 and 0.6% (v/v) Resolyte pH 4-8. The samples

(80 μ l intercellular fluid) were precipitated by adding 5 volumes of cold acetone overnight at -20°C . After centrifugation ($11\,000 \times g$, 10 min, 4°C), the acetone was discarded and the pellet was dried under vacuum and resuspended in 5 μ l distilled water, and then mixed with an equal volume of sample buffer (60% glycerol, 3.2% Pharmalyte pH 3-10 and 0.8% Resolyte pH 4-8) and spun at $10,000 \times g$ for 5 min before loading. The samples were applied into the bottom of the wells and filled the space above the samples in the wells with 1% Pharmalyte pH 3-10 and 5% (w/v) sucrose solution which protect the proteins from harsh pH of the catholyte solution in the upper buffer chamber. The gels and electrophoresis buffers were precooled at 4°C . The gels were electrophoresed at room temperature 200 V for 1.5 h and 400 V for 1.5 h. Upper and lower chamber running buffers were 20 mM sodium hydroxide and 10 mM phosphoric acid, respectively. Five μ l of IEF standards (*BIO-RAD*), ranging from pI 4.45 to 9.6, was loaded in one well and coelectrophoresed to estimate the pIs of the proteins. For silver stained gel, the IEF markers were diluted at 1: 500 in sample buffer.

2.9.5 Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE)

Two dimensional resolution of proteins was carried out essentially according the methods of O'Farrell (1975), Hochstrasser et al. (1988a) and Jeknic and Chen (1999).

2.9.5.1 Sample preparation

The intercellular fluid from treated leaves or control was precipitated by addition of 5 volumes of 0.7% (v/v) 2-mercaptoethanol in cold acetone and incubated at -20°C overnight. After centrifugation at $15,000 \times g$ for 15 min at 4°C , the supernatant was discarded and the pellet was dried under vacuum at room temperature. The protein was resuspended in a lysis buffer (O'Farrell 1975) containing 9.5 M urea, 2% (w/v) CHAPS, 0.4% (v/v) Pharmalyte pH 3-10, 1.6% (v/v) Resolyte pH 4-8, and 5% (v/v) 2-mercaptoethanol. The sample was thoroughly mixed and incubated on ice for 30 min. The insoluble material in solutions was removed by centrifugation ($14,000 \times g$ for 15 min at room temperature). Protein concentrations ($\mu\text{g}/\mu\text{l}$) in the supernatant and standard bovine serum albumin solutions were determined by the modified

Bradford method (Ramagli and Rodriguez 1985). All samples were loaded onto IEF gels (the first dimension of the 2D-PAGE) immediately after preparation. In order to estimate the molecular weights and pIs of induced proteins, 4 μ l of 2D SDS-PAGE standards (*BIO-RAD*) was also included in the supernatant of one of three samples, in each treatment.

2.9.5.2 Isoelectric focusing in the first dimension

The first dimension was performed in the PROTEANTM II cell (*BIO-RAD*). Proteins were separated in 1 \times 145 mm tube gels of polyacrylamide by isoelectric focusing (IEF). Twenty micrograms of total protein in 20 μ l of lysis buffer was loaded at the cathodic end of each tube gel containing 8 M urea, 4% (w/v) acrylamide, 0.4% (v/v) Pharmalyte pH 3-10, 1.6% (v/v) Resolyte pH 4-8, then overlaid with 10 μ l covering buffer consisting 7.5 M urea, 0.17% Pharmalyte pH 3-10, 0.67% Resolyte pH 4-8, and finally the tube was filled with catholyte buffer. The gels were run at 200 V for 20 min, then at 400 V for 20 min and then at 900 V for 20 h, with 50 mM sodium hydroxide as a cathodic buffer and 50 mM phosphoric acid as an anodic buffer. After IEF run, 1 μ l of concentrated bromphenol blue containing 50% (v/v) aqueous glycerol saturated with bromphenol blue was loaded on the top of each gel. The dye marked the basic end of the gel and was used as a tracking dye in the second dimensional SDS-PAGE. Gels were then extruded, equilibrated in 500 μ l transfer buffer (0.5 M Tris-HCl pH 6.8, and 0.4% SDS) in Eppendorf tubes and stored at -80°C until use.

2.9.5.3 SDS-PAGE in the second dimension

The second dimension was carried out by SDS-polyacrylamide gel electrophoresis (Laemmli 1970) in a vertical slab unit, PROTEANTM II slab cell (*BIO-RAD*) at room temperature. IEF gels were thawed at room temperature and equilibrated with a fresh transfer buffer (0.5 M Tris-HCl pH 6.8, and 0.4% SDS), then placed upon 15% polyacrylamide slab gels (165 \times 180 \times 1.5 mm) which were basically the separating gel of SDS-PAGE without a stacking gel. For estimation of molecular weights of induced proteins, low molecular weight standards (*BIO-RAD*) were also used. Markers were prepared as following. Molecular weight markers were added at a

dilution of 1:100 (v/v) in SDS sample buffer containing 1% (w/v) agarose and boiled for 5 min in a water bath. The hot solution was poured into a glass tube (ϕ 1 mm). After solidification, the gel was extruded, cut into 5 mm pieces, and then stored at -80°C . The gel piece was placed at the acidic side of the IEF gel on the top of the separating gel. The upper (cathode) buffer was 50 mM Tris, 195 mM glycine, and 0.1% (w/v) SDS; the lower (anode) buffer was the same as cathodic buffer except the inclusion of 0.02% (w/v) sodium azide. The gels were run at 35 mA constant current per gel until the tracking dye reached the bottom of the gel.

2.9.6 Staining protein gels

2.9.6.1 Coomassie staining

After electrophoresis, the gels were stained overnight with a solution containing 0.1% (w/v) Coomassie Brilliant Blue R₂₅₀, 50% (v/v) methanol and 10% (v/v) acetic acid. They were then destained through several changes of a solution consisting of acetic acid: methanol: water (1:2:17, v/v/v) and stored in 7% (v/v) acetic acid.

2.9.6.2 Silver staining

Alternatively, the gels were silver stained according to the method of Hochstrasser et al. (1988b). Following electrophoresis, the separating gels were fixed in a solution of 50% methanol and 10% acetic acid for 1 h. After wash in dH₂O four times (5 min each wash), they were incubated in 10% (v/v) glutaraldehyde solution for 30 min. Extensive washes with dH₂O for 4×15 min were then performed to completely remove the glutaraldehyde. Following overnight wash in dH₂O, the water was drained off and a freshly made silver staining solution containing 0.7% (w/v) AgNO₃, 1% (v/v) ammonia solution and 0.08% (w/v) NaOH was added and stained for 15 min. After staining, the gels were rinsed with dH₂O for 4×5 min and subsequently were placed in a developing solution consisting of 0.01% (w/v) citric acid and 0.1% (v/v) formaldehyde. All steps were carried out with gentle shaking. When the bands became visible within 10 min, the development process was stopped with 5% (v/v) acetic acid and the stained gels were immediately photographed.

For IEF slab gels, fixation was first carried out in 20% (w/v) trichloroacetic acid (TCA) for 1 h. The gels were then incubated in 5% (v/v) glutaraldehyde in 0.5 M sodium phosphate buffer (pH 7.5) for 30 min at room temperature. After fixation, the gels were washed and stained as described above.

2.10 Assay of enzyme activities

2.10.1 Assay of β -1,3-glucanase activity

Activity of β -1,3-glucanase in intercellular fluid was assayed by measuring the rate of reducing sugar production using laminarin as the substrate as described by Boller (1992) with slight modification. The reaction mixture (250 μ l), containing 50 μ l of 0.1 M sodium acetate buffer (pH 5.5), 50 μ l of 1% reduced laminarin or 50 μ l H₂O as enzyme blank, 20 μ l of undesalted ICF at a dilution of 1:10 with distilled water and 130 μ l of 10 mM sodium acetate (pH 5.5) in a 10 ml test tube, was incubated at 37°C for 30 min. After incubation, the tube was placed on an ice-bath and 2 ml of the basic copper reagent [4% (w/v) Na₂CO₃, 1.6% (w/v) glycine, 0.045% (w/v) CuSO₄·5H₂O] and 2 ml of 0.12% (w/v) fresh neocuproin solution was added to each tube. The test tubes were then heated at 100°C for 12 min. After cooling, 3 ml distilled water was added to each tube. The resulting reducing sugar (glucose) released from laminarin by β -1,3-glucanases was determined by measuring absorbance at 450 nm in a spectrophotometer (Novaspec II, Pharmacia Biotech). Glucanase activity was expressed in nkatal mg⁻¹ protein. One nkatal was defined as the enzyme activity catalysing the formation of 1 nmol of glucose per second.

2.10.2 Assay of chitinase activity

Chitinase activity was assayed colourimetrically with CM-Chitin-RBV as the substrate according to Wirth and Wolf (1990). The reaction mixture (total volume of 0.4 ml) consisted of 5 μ l of ICF extract, 195 μ l of water, 0.1 ml of 0.2 M sodium acetate buffer, pH 5.0 and 0.1 ml of CM-Chitin-RBV solution. After thoroughly mixing, the solution was incubated at 37°C for 10 min. The reaction was stopped by addition of 0.4 ml of 0.5 M HCl and left on ice for 10 min. The undigested substrate

was precipitated by centrifugation ($12000 \times g$ for 5 min at 4°C). The supernatant was used for the colourimetric determination at 550 nm. The control for each extract was prepared similarly, only enzyme extract was added after addition of HCl. Chitinase activity was expressed in units (U) mg^{-1} protein. One unit was defined as the enzyme activity catalysing the formation of 1 nmol N acetyl glucosamine (GlcNAc) per minute.

2.10.3 Assay of peroxidase activity

Peroxidase activity of ICF extract was assayed according to Rao et al. (1996) with minor modification. Guaiacol was used as a substrate. The reaction mixture (1 ml in an Eppendorf tube) consisted of 0.1 M potassium phosphate buffer pH 6.5, 16 mM guaiacol and 5 μl of 10% H_2O_2 . The ICF extract (5 μl) was added to initiate the reaction at 25°C for 5 min. Peroxidase activity was expressed as change in 470 nm per min per microgram of protein.

2.10.4 Assay of mannosidase activity

Mannosidase, a marker enzyme of the vacuole of plant cells, was used to check the contamination of intercellular fluid by cytoplasmic proteins. The α -mannosidase activity in ICFs and intracellular extracts was measured spectrophotometrically using 4-nitrophenyl- α -D-mannopyranoside as substrate according to Boller and Kende (1979) and Heitz et al. (1991). After ICF was collected, the infiltrated leaf tissue was frozen in liquid nitrogen and ground to a fine powder with a mortar and pestle. To the powder, four volumes of cold 0.1 M sodium acetate (pH 5.2) containing 5 mM CaCl_2 , 15 mM β -mercaptoethanol and 0.7% (w/v) charcoal were added. The homogenate was centrifuged at $12500 \times g$ for 30 min. The supernatant was used to assay mannosidase activity. The reaction mixture containing 0.5 ml of 0.1% (w/v) 4-nitrophenyl- α -D-mannopyranoside in 0.1 M sodium acetate buffer, pH 5.2 and 10 μl of ICF extract or intracellular extract was incubated at 37°C for 30 min. The enzyme reaction was terminated by adding 0.5 ml of 10% (w/v) Na_2CO_3 . α -Mannosidase activity was expressed as change in A_{405} per hour per g fresh weight under the assay conditions.

2.11 Detection of enzymes after PAGE or IEF

ICFs were concentrated by adding 5 volumes of cold acetone overnight at -20°C and then centrifuged at $12000 \times g$ for 10 min. The pellets were dried under vacuum at room temperature and dissolved in distilled water. The insoluble material in protein solutions was removed by centrifugation at $12000 \times g$ for 5 min before electrophoresis and isoelectric focusing.

2.11.1 Detection of β -1,3-glucanase isoforms

β -1,3-glucanase isoforms were detected according to the method of Pan et al. (1991). After non-denaturing PAGE or IEF, gels were rinsed with distilled water and incubated in 50 mM sodium acetate buffer (pH 5.2) for 10 min. The gels were then incubated at 40°C for 2 h in 50 mM sodium acetate (pH 5.2) containing 0.6% (w/v) laminarin. After incubation, the gels were fixed in water: methanol: acetic acid (5:5:2) for 5 min and washed three times with distilled water. The gels were then transferred to 0.15% (w/v) 2,3,5-triphenyl-tetrazolium chloride in 1 M NaOH solution. The containers with gels were heated in boiling water bath until red bands appeared after about 5 min. After staining, gels were put in 7.5% acetic acid and stored at 4°C .

2.11.2 Detection of chitinase isoforms on the overlay gels

Chitinase activity was detected as described by Pan et al. (1991). Following non-denaturing PAGE or IEF, the gels attached to the supporting glass plates were incubated in 0.1 M sodium acetate buffer (pH 5.0) for 15 min. The separating gels were then covered with 7.5% polyacrylamide overlay gels (0.75 mm thick, attached to another supporting glass plate) containing 0.04% (w/v) glycol chitin in 0.1 M sodium acetate (pH 5.0). The gels were incubated at 37°C for 2 h in a plastic container under moist conditions. Following incubation, the overlay gels were stained for 5 min in freshly prepared 0.01% (w/v) fluorescent brightener 28 in 0.5 M Tris-HCl (pH 8.9) at room temperature, then destained in distilled water overnight at 4°C . Chitinase isoforms were visualised by placing the overlay gels on an UV transilluminator.

2.11.3 Detection of peroxidase isoforms

Peroxidase isoforms were visualised following the method described by Hammerschmidt and Kuc (1982). After non-denaturing PAGE or IEF, the gels were incubated in 0.1 M sodium acetate buffer pH 4.5 containing 1 mM o-dianisidine for 2 h at 25°C and then briefly rinsed in distilled water. The green bands appeared immediately after transferring to 1% (v/v) H₂O₂. However, they quickly changed to orange-brown colour and became fainter within an hour. Therefore, photos of the gels were immediately taken after the appearance of green bands.

2.12 Identification of the induced proteins

2.12.1 Production of polyclonal antibodies

Polyclonal antibodies were raised against intercellular fluid proteins extracted from leaves of rose shoots var. 'Iris Gee' 5 days after treatment with 50 µM BTH or 7 days after inoculation with *D. rosae*. New Zealand white rabbits were immunised by monthly injection with 1 ml ICF diluted with an equal volume of Freund's complete adjuvant for two times. Two booster injections were then given at monthly intervals subcutaneously. The rabbits were bled 2 weeks after the fourth injection. The serum was obtained by centrifugation and stored at -20°C. Prior to antigen injection, a blood sample was taken to be used as preimmune serum.

2.12.2 Purification of polyclonal antibodies by adsorption

The procedure for purifying polyclonal antibodies was carried out following the method of Choi et al. (1987). Nitrocellulose membrane disks (32 mm in diameter) were soaked in distilled water for 5 min and dried at room temperature. The disks were then saturated with ICF extracted from leaves of rose shoots pretreated with water. After membranes were dried, the disks were incubated in the antibodies diluted (1:20) with block buffer (10 mM Tris-HCl pH 7.5, 0.15 M NaCl, 0.1% Tween-20, 3% bovine serum albumin) for 30 min. This procedure was repeated five times.

2.12.3 Western blotting

After SDS-PAGE on a 15% acrylamide gel, mini gels were immediately soaked for 30 min in a transfer buffer containing 25 mM Tris, 192 mM glycine, and 20% (v/v) methanol. The proteins were electrophoretically transferred from polyacrylamide gels to nitrocellulose membranes (0.2 μ m pore size, Schleicher & Schuell, Germany) or polyvinylidene difluoride (PVDF) membranes (0.2 μ m, *BIO-RAD*) following the procedure of Towbin et al. (1979). PVDF membranes need to be prewetted with methanol for three seconds and were then transferred to distilled water for 2 min to rinse off the methanol. The membranes were subsequently incubated in the transfer buffer. Electroblotting was carried out on a TE 70 semidry transfer unit (Hoefer Scientific Instruments, San Francisco, USA) at 30 mA for 1 h at room temperature, according to the manufacturer's instructions. After blotting was completed, the lane of marker proteins was cut and visualised by staining with Coomassie blue R-250. The non-specific binding sites on the membranes were blocked for 60 min in blocking solution containing TBST (10 mM Tris-HCl pH 7.5, 0.15 M NaCl, 0.05% Tween 20) supplemented with 5% (w/v) non-fat dry milk and washed in TBST three times for 10 min each. Nitrocellulose blots were then incubated with the primary polyclonal antibodies at a dilution (see the next page) in TBST plus 1% non-fat milk powder for overnight at room temperature. Afterwards, the blots were washed extensively with TBST three times as described above and incubated with secondary antibody against rabbit IgG conjugated with alkaline phosphatase (Boehringer Mannheim) at a dilution of 1:1250 with blocking buffer for 4 h. Thereafter, the membranes were rinsed three times for 10 min each in a large volume of TBST. The bands were visualised by placing the blots in 10 ml of alkaline phosphatase buffer (100 mM Tris-HCl pH 9.5, 100 mM NaCl and 5 mM MgCl_2) containing 66 μ l NBT (5% nitro blue tetrazolium in 70% dimethylformamide) and 33 μ l BCIP (5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt in 100% dimethylformamide). The reaction was stopped by removing the developer solution and rinsing the membrane with distilled water.

Alternatively, 5 μ l of prestained SDS-PAGE standards were loaded on the gel to monitor the protein transfer by electrophoretically blotting. The prestained low-

molecular-range SDS-PAGE standards (*BIO-RAD*) consist of phosphorylase B 111 kDa, bovine serum albumin 73 kDa, ovalbumin 47.5 kDa, carbonic anhydrase 33.9 kDa, soybean trypsin inhibitor 28.8 kDa, and lysozyme 20.5 kDa.

Polyclonal antibodies raised against intercellular fluid extracted from leaves 5 days after 50 μ M BTH treatment or 7 days after inoculation with *D. rosae* were diluted at 1: 2000. Antisera raised against the four major classes of tobacco PR proteins were a generous gift from Dr. Kuffmann (Centre National de la Recherche Scientifique, Strasbourg, France). The antiserum was diluted at 1: 500 for PR-1c (PR-1 family) or at 1: 5000 for PR-N (PR-2 family), PR-Q (PR-3 family), and PR-S (PR-5 family), respectively.

2.12.4 Purification and microsequencing of the induced proteins

For N-terminal amino-acid sequencing, the procedure of 2D-PAGE was the same as the stated above except that purified reagents were used and gel solutions were filtered through 0.45 μ m filter, and the upper running buffer was supplemented with 0.1 mM thioglycolate to avoid N-terminal blocking. In addition, a greater glass tube with 2.5 mm internal diameter was used for the first dimension IEF and thicker gel for the second dimension SDS-PAGE. One hundred μ g of intercellular proteins, extracted from leaves of *in vitro* rose shoots 5 days after BTH treatment, were dissolved in the 2D sample buffer before being loaded onto the first dimensional IEF gel. After separation by 2D-PAGE, the proteins on the gels were transferred to PVDF membranes (*BIO-RAD*) using a TE 70 semi-dry transfer unit at 0.8 mA/cm² according to the manufacturer's instruction. After transfer, the membranes were washed with distilled water three times (5 min each). Electroblots were then stained with 0.025% Coomassie Blue R-250 in a 40% methanol solution for 5 min and destained with 50% methanol solution until the colour in the background disappeared. The membrane was thoroughly washed in distilled water for 10 min and then dried completely. The spots of interest were excised from the PVDF membrane along the borders of the protein spots and analysed by Protein Microchemistry Facility using a standard sequencing procedure at the Biochemistry Department of Otago University, Dunedin, New Zealand.

2.13 Experimental design, statistics and data analysis

2.13.1 Experimental design

Experiments were designed as a randomised complete block with three replicates. All were repeated at least twice with 24-30 shoots per treatment.

2.13.2 Disease analysis

The chi-squared test was used to statistically determine gall formation frequency of shoots and percentage of germination of *D. rosae* conidia. Analysis of variance (ANOVA) was conducted to evaluate the significance of treatments on the size of gall formation and the severity of blackspot disease. Differences between treatments were tested by Tukey's multiple range comparison test with significance level at $P < 0.05$ or at $P < 0.01$. All statistical analyses were performed using the SPSS for Windows statistical software package (SPSS Inc., Version 8.0, 1998).

2.13.3 Gel analysis

The molecular weights of induced bands were calculated from three independent SDS gels based on their mobilities in the gel in relation to that of standard proteins (SDS-PAGE lower molecular weight standards or 2D-SDS-PAGE standards, *BIO-RAD*). Three two-dimensional gels per treatment were run and visually examined. In order to compare the spot differences between treatments and control, individual spots were investigated based on molecular weight markers, isoelectric points, prominent global spots present on all gels, neighbourhood spot patterns, and spot colour as described by (Thompson-Coffe et al. 1992, Jeknic and Chen 1999). Only those changes appearing in all gels of each set of samples were recorded. The pIs of proteins of interests in this studies were estimated from a regression equation of the standard proteins versus distance migrated on IEF gels.

2.13.4 Enzyme activity assay

Each experiment was repeated twice with similar results. All values are shown as the means \pm standard errors of triplicate results obtained in one experiment. The data were statistically analysed by the *t*-test at 5% level of significance.

Chapter 3

RESULTS

3.1 Induction of disease resistance in response to BTH treatment

3.1.1 Effect of BTH on the *in vitro* growth of *D. rosae* and *A. tumefaciens*

3.1.1.1 *D. rosae*

It has been reported that BTH does not have any apparent antifungal effects on a range of fungal pathogens *in vitro* (Friedrich et al. 1996, Benhamou and Belanger 1998b, Ishii et al. 1999). The test on whether BTH has any direct antifungal activity on *D. rosae* was carried out *in vitro* in microtitre plates at 21°C in the dark. Observation under microscope revealed that there was no discernible difference in the growth of the fungus in the presence or absence of 50 µM or 500 µM BTH after 6 days from the start of incubating *D. rosae* conidia (Plate 3.1).

3.1.1.2 *A. tumefaciens*

It was observed that there was no distinct difference between the growth of *A. tumefaciens* cultured on LB medium and LB medium containing 50 µM BTH or 0.5 mM BTH (Plate 3.2).

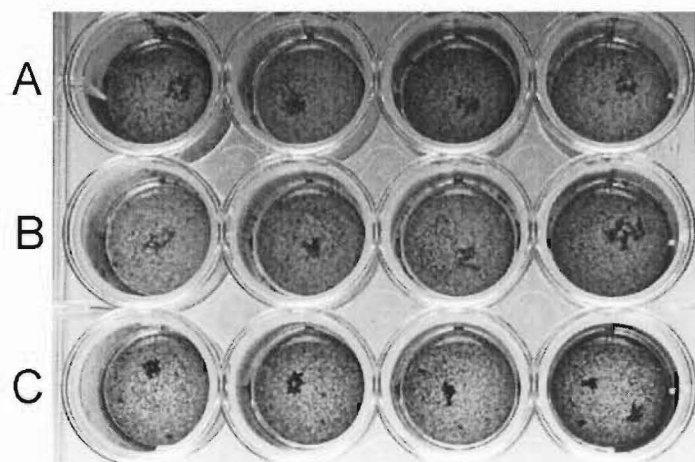


Plate 3.1 Effect of BTH on the *in vitro* growth of *D. rosae*.

Fifty microlitres of conidial suspension (total 500 conidia) were added to the wells containing potato dextrose broth supplemented with (A) 0 μM BTH, (B) 50 μM BTH and (C) 500 μM BTH. The photograph was taken after 6 days from the start of incubating *D. rosae* conidia at 21°C in the dark. Mycelia were stained with lactophenol cotton blue.

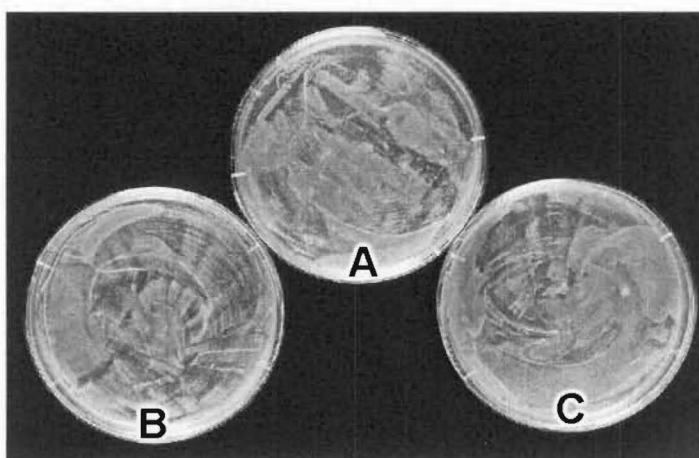


Plate 3.2 Effect of BTH on the *in vitro* growth of *A. tumefaciens*.

(A) BTH-free LB medium, (B) LB medium + 50 μM BTH and (C) LB medium + 500 μM BTH. The photograph was taken after 2 days from plating *A. tumefaciens* at 26°C in the dark.

3.1.2 Effect of BTH treatment on *in vitro* rose shoots

When *in vitro* rose shoots (var. Iris Gee) were dipped into different concentrations of BTH (ranging from 5 μ M to 5 mM), no visible adverse effect was observed on the treated shoots after 7 days from the application. BTH at a concentration below 0.5 mM had no effect on the growth and morphology of shoots compared to the shoots treated with water. However, the growth of shoots treated with a concentration higher than 0.5 mM was retarded, particularly the internode lengths of stems and the sizes of leaves (Plate 3.3). Later on in the experiment, lower leaves of the treated shoots turned yellow.

When rose plants grown in pots were sprayed with BTH up to 0.5 mM, no phytotoxic effect was observed. Only application of this chemical at the concentrations of 5 mM and higher seemed to cause the newly expanded leaves to become smaller compared with those of control (data not shown).

3.1.3 Rose shoot protection by BTH

3.1.3.1 Induced resistance against *D. rosae*

In a preliminary experiment, the susceptibility of five rose varieties to *D. rosae* (black spot) was evaluated by the method of inoculation with the pathogen on detached leaves of pot plants in Petri dishes. Seven days after inoculation, the disease severity on a 0-7 scale for the rating of each leaf was determined. It was found that no variety showed full resistance. However, the var. Alexander demonstrated lower susceptibility than the others.

In addition, a quick and simple method to test the resistance of *in vitro* rose shoots to *D. rosae* was developed under *in vitro* conditions. Rose shoots were inoculated by submerging them into a conidial suspension of *D. rosae* under otherwise aseptic conditions. The shoots were subsequently transferred to jars containing 0.8% agar for the disease development. Three days after inoculation, small but visible blackspots appeared on the basal parts of upper leaf surfaces. At day 5, the infected leaves were fully covered with small black lesions. These lesions expanded and were surrounded by extensive chlorosis of some leaf tissue after 7 days from the inoculation (Plate 3.4). By 10 days, the heavily infected leaves turned yellow and abscised from the shoots. However, the noninoculated leaves did not show any disease symptoms.

In order to test whether BTH induces blackspot resistance in rose, *in vitro* shoots of var. Iris Gee were dipped into BTH solutions at different concentrations or distilled water as the control, and then kept on MS medium for 4 days before inoculation with *D. rosae*. To avoid a potential influence of BTH and the effect of the MS medium around the stems on the growth of fungus, the shoots were first washed with sterile distilled water before submerging them into a conidial suspension of *D. rosae* under otherwise aseptic conditions. Three days after inoculation, the germination of conidia was observed under a UV microscope. Most germ tubes originated from one end of the spore (Plate 3.5). In very few cases, the conidia germ tubes occurred either from both ends or from the area near the conidial septum in agreement with the observation of Wiggers et al. (1997). However, there were no significant differences in the percentages of spore germination 3 days after inoculation among the control and BTH treatments (Table 3.1).

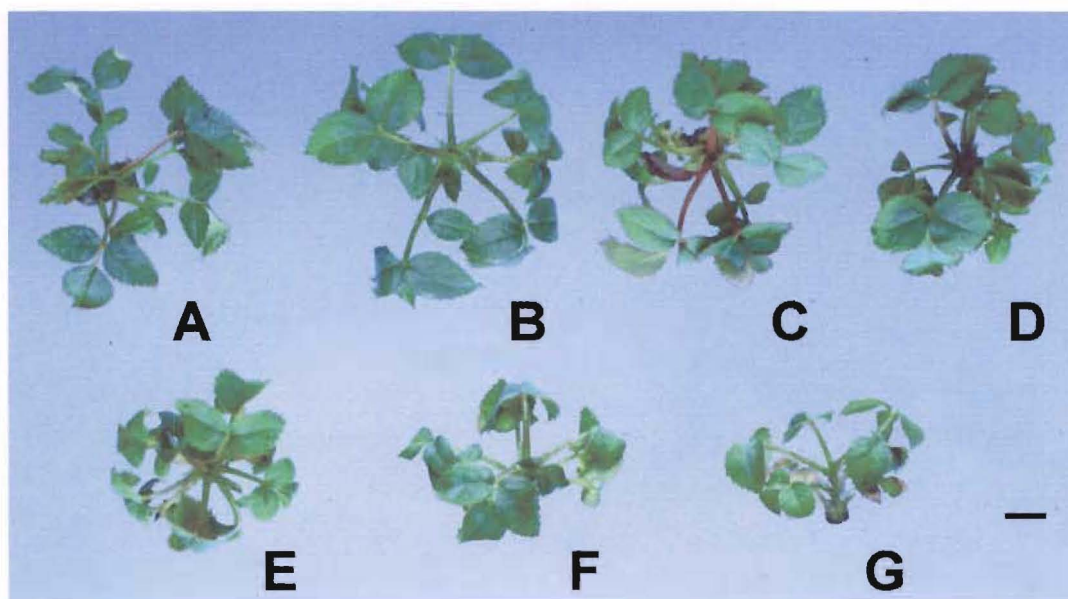


Plate 3.3 Effect of BTH treatment on the growth of *in vitro* rose shoots (var. Iris Gee).

BTH was applied by dipping whole *in vitro* rose shoots into the chemical at different concentrations for a few seconds before returning them to shoot growth medium. The photograph was taken after 7 days from the start of the treatments, scale bar = 1 cm. Rose shoots were treated with distilled water (A), 5 μM BTH (B), 50 μM BTH (C), 50 μM BTH^f (D), 500 μM BTH (E), 2.5 mM BTH (F), and 5 mM BTH (G). All solutions were autoclaved except BTH^f, which was filter sterilised.

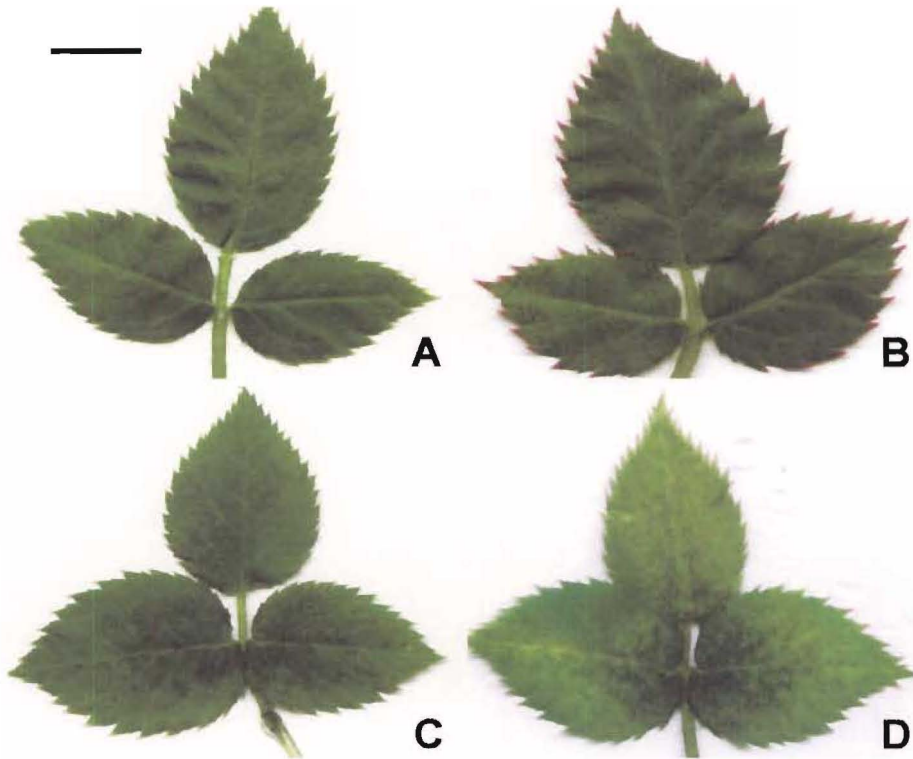


Plate 3.4 The disease development on the leaves of *in vitro* rose shoots (var. Iris Gee) after inoculation with *D. rosae*.

Rose shoots grown *in vitro* were inoculated by dipping them into a conidial suspension of *D. rosae* under otherwise aseptic conditions. The typical infected leaves were detached and photographed, at 1 day (A), 3 days (B), 5 days (C), and 7 days (D) after inoculation. The scale bar = 5 mm. The pictures were taken from one experiment, but similar results were obtained from other experiments.

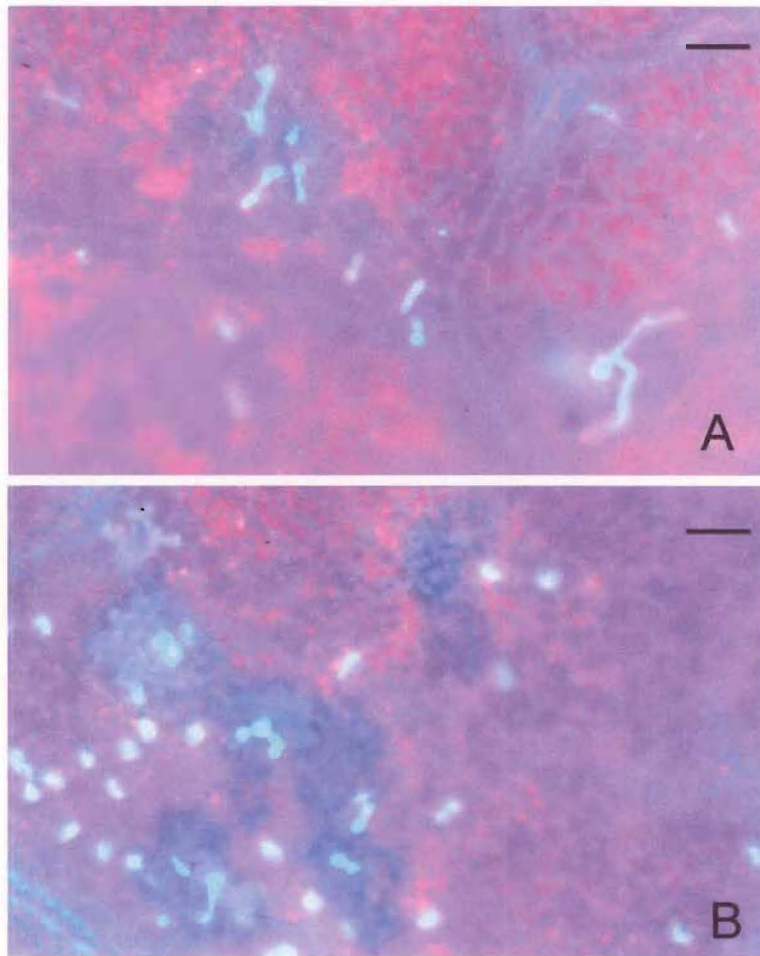


Plate 3.5 Epifluorescence light microscopic observations on the germination of *D. rosae* conidia on leaves of *in vitro* rose shoots (var. Iris Gee) 3 days after inoculation.

Germination and growth of *D. rosae* conidia on water-treated leaves (A), and BTH-treated leaves (B). Scale bars = 50 μm .

Table 3.1 Percent germination of *D. rosae* conidia on the leaves of *in vitro* rose shoots (var. Iris Gee) that had been previously treated with water or different concentrations of BTH

Treatment with BTH (μM)	Percentage of germination *
0	42.0 ± 7.44
5	38.1 ± 5.69
50^f	36.9 ± 5.78
50	34.4 ± 3.32
500	33.2 ± 3.43

* Mean values \pm standard errors. It was scored based on the count of 300 conidia on the leaves of three shoots in each treatment after 3 days from inoculation with *D. rosae* conidia.

^f The BTH solution was filter sterilised before use.

Disease symptoms, as evident by percent infected leaf area per shoot treated with different concentrations of BTH and water control, were evaluated after 7 days. No significant difference was observed between the low concentration BTH (5 μM) and the water-treated control. However, shoots treated with BTH at concentrations ≥ 50 μM displayed significant reduction in disease severity compared with the control (Figure 3.1 and Plate 3.6).

Upon closer examination, blackspots appeared to be widespread and caused many large lesions which were surrounded by spreading chlorosis on the infected leaves of the water-treated shoots (Plate 3.6A). In contrast, the BTH-treated shoots showed fewer and smaller lesions with less chlorosis (Plate 3.6C and D). Although the best protection against *D. rosae* was observed in the shoots treated with 0.5 mM BTH (Plate 3.6D), this high concentration of BTH retarded slightly the stem growth of the treated shoots, indicating possible phytotoxic effects. However, this could be a property of *in vitro* grown shoots, often lacking surface protective cuticles. This is consistent with another experiment showing that spraying one-month old rose pot plants at this concentration had no noticeable negative effects.

The development of disease symptom on the leaves of *in vitro* rose shoots treated with 50 μ M BTH or water over 7 days after inoculation with *D. rosae* was shown in Figure 3.2. Visible necrotic lesions first appeared on the leaves of rose shoots 3 days after inoculation. After this, the protection effect of BTH became more apparent. By 7 days after inoculation with *D. rosae*, disease severity was greatly reduced on the rose shoots pretreated with BTH.

Stereo-microscopic examination of the infected leaves staining with trypan blue showed that at 1 day after inoculation, both the BTH-treated and water-treated (control) leaves had few necrotic cells (Plate 3.7). Three to seven days after inoculation, the water-treated leaves showed many large necrotic areas and the development of hyphae from the primary hyphae, while the BTH-treated leaves had many smaller necrotic areas and less visible hyphae. After 7 days from inoculation, larger necrotic areas and more newly formed conidia were observed by staining with trypan blue on the control leaves (Plate 3.8A and C) than on the BTH-treated leaves (Plate 3.8B and D). This indicates that pretreatment of *in vitro* rose shoots with BTH reduced the rate and extent of *D. rosae* colonisation compared with control shoots treated with water. This result also correlates well with the development of numerous blackspots on the water-treated leaves (Plate 3.6A).

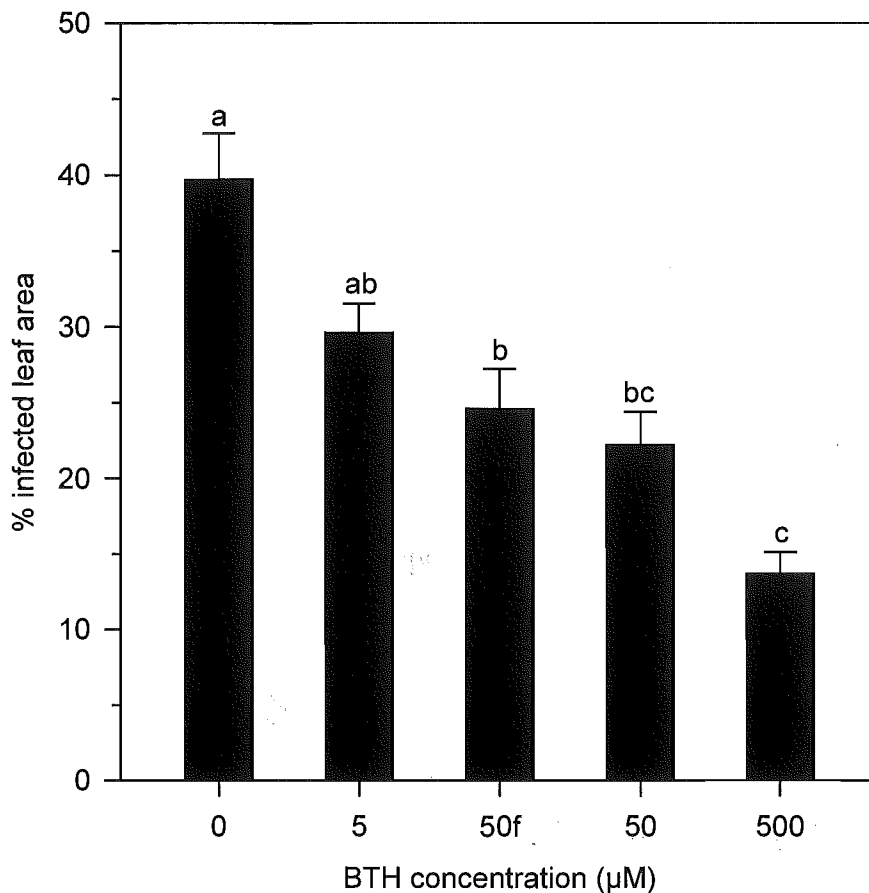


Figure 3.1 Effect of BTH pretreatment on the severity of blackspot disease of *in vitro* rose shoots (var. Iris Gee).

BTH was applied by dipping whole *in vitro* rose shoots (var. Iris Gee) into either the chemical at different concentrations or water 4 days before challenge inoculation. The shoots were subsequently inoculated by submerging them into a conidial suspension of *D. rosae*. Percentages of infected leaf areas are means of 24 shoots per treatment after 7 days from inoculation. All solutions were autoclaved except 50f, which was filter sterilised. Values with different letters are significantly different using Tukey's method, $P < 0.05$.

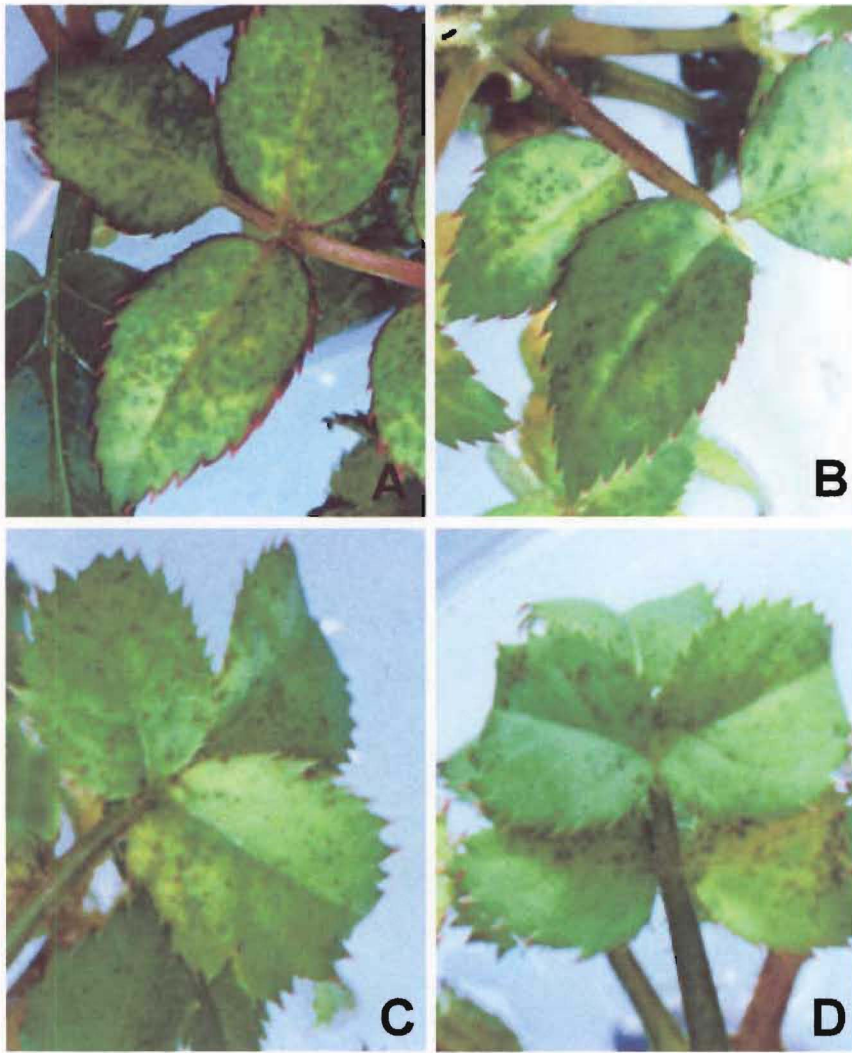


Plate 3.6 Protection of *in vitro* rose shoots (var. Iris Gee) against *D. rosae* by BTH application.

BTH was applied by dipping *in vitro* rose shoots into either the chemical at different concentrations or water 4 days before challenge inoculation. The whole shoots were subsequently inoculated by submerging them into a conidial suspension of *D. rosae*. Shoots were pretreated with water (A), 5 μM BTH (B), 50 μM BTH (C), and 500 μM BTH (D). The photographs were taken after 7 days from the inoculation.

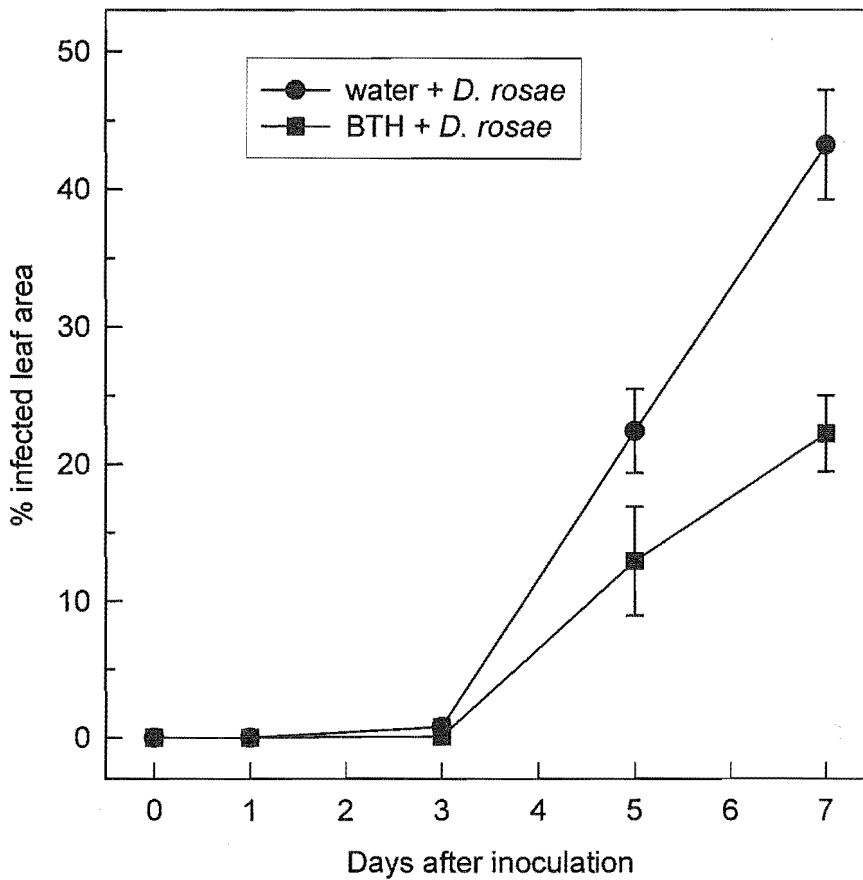


Figure 3.2 Effect of BTH pretreatment on the disease development of blackspot of *in vitro* rose shoots (var. Iris Gee) over a period of 7 days.

Rose shoots were first submerged into a 50 μ M BTH solution or water for a few seconds 4 days before challenge inoculation. Following this, the shoots were inoculated by dipping them into a conidial suspension of *D. rosae* as well. Percentages of infected leaf areas are means of 24 shoots per treatment at various times after inoculation. Each bar represents a mean \pm standard error from three replicates. (●) water + *D. rosae*; (■) BTH + *D. rosae*.

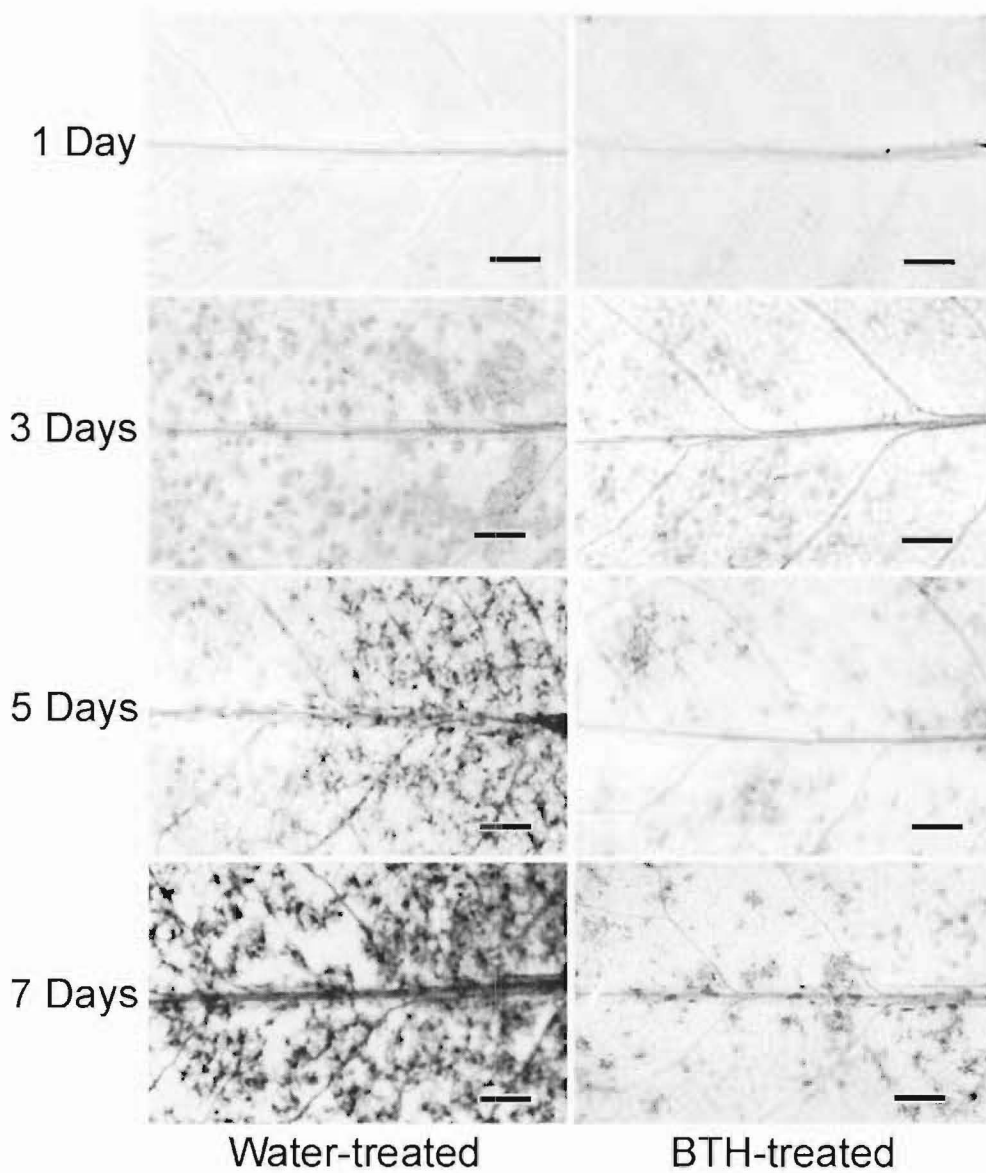


Plate 3.7 Stereo-microscopic observations of leaves of *in vitro* rose shoots (var. Iris Gee) stained with trypan blue after inoculation with *D. rosae*.

At various times after inoculation with *D. rosae*, indicated on the left, the typical infected leaves treated with water or 50 μ M BTH were detached and stained with trypan blue (scale bars = 1 mm). The pictures were taken from one experiment, but similar results were obtained from other replicate experiments.

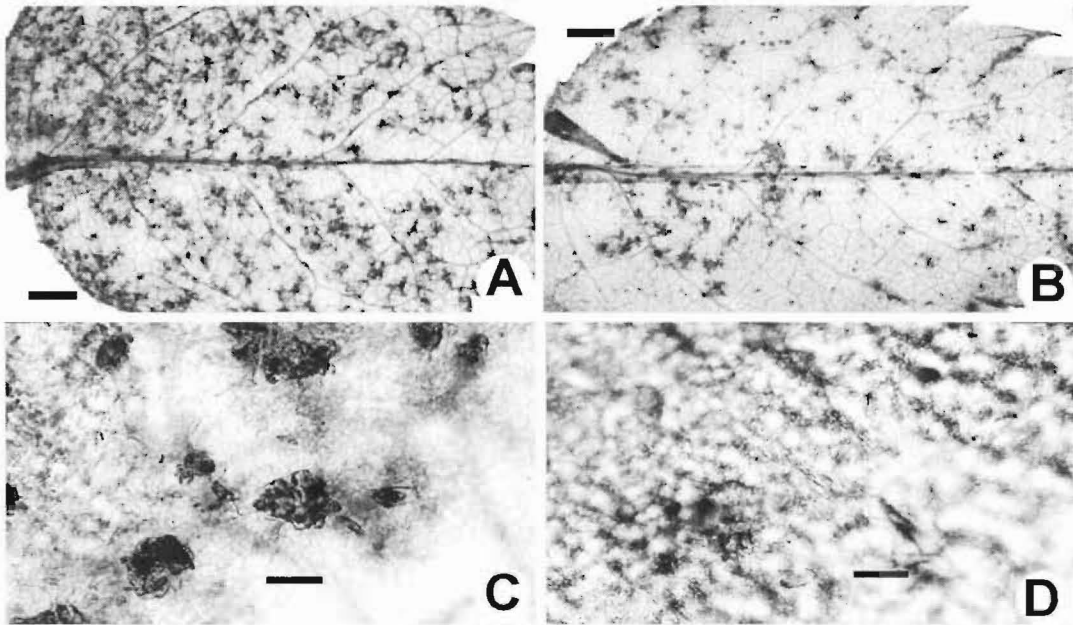


Plate 3.8 Effect of BTH on sporulation intensity of *D. rosae* on the leaves of *in vitro* rose shoots (var. Iris Gee) 7 days after inoculation.

(A) and (C) water-treated rose leaf, (B) and (D) rose leaf treated with 50 μM BTH.
Scale bars = 1 mm in (A) and (B); Scale bars = 100 μm in (C) and (D).

3.1.3.2 Induced resistance against *A. tumefaciens*

Preliminary experiments were carried out to evaluate the susceptibility of three rose varieties to *A. tumefaciens* strain C58 by determining the frequencies and sizes of crown gall formation after one month from inoculation of *in vitro* rose shoots. Different responses of the shoots to *A. tumefaciens* were found among the rose varieties. 'Madam Isaac Pieriere' was more susceptible to *A. tumefaciens* showing 70% gall formation, as compared with var. 'Iris Gee' and 'Alexander', showing 25% and 40% gall formation, respectively. Therefore, var. 'Madam Isaac Pieriere' was used in the following experiments to test whether a pretreatment with BTH could induce resistance against *A. tumefaciens*. Furthermore, since the previous experiments (Figure 3.1 and Plate 3.6) demonstrated that a pretreatment with 50 μ M BTH had some protective effects against *D. rosae*, here, this concentration of BTH was chosen for experiments with attempted infection by agrobacteria to test whether BTH-induced resistance was also effective against *A. tumefaciens*.

Four days after application of BTH, the shoots were inoculated with *A. tumefaciens*. The calli at the inoculated sites were visible after 6 days from inoculation. The galls at this stage could not be distinguished from calli. However, they developed more rapidly than calli at later stage. The galls exhibited light green to light brown colours.

It has been reported that there were no significant difference among upper, intermediate and lower inoculation sites on rose plants in response to *Agrobacterium* infection (Cervera et al. 1998). Therefore, in the present experiments, each inoculated site was considered to be an independent replicate. The results clearly showed that the larger sizes and higher frequency of galls were observed in water-treated shoots,

whereas the shoots treated with BTH resulted in significantly decreased frequency of gall formation and gall sizes, as shown in Table 3.2 and Plate 3.9.

In order to clarify if the formation of galls was an effect of *A. tumefaciens* infection, the strain C58 with the recombinant plasmid pIG 121 (Ohta et al. 1990) was used to inoculate rose shoots as before. This strain harbours a T-DNA gene construct that permits the expression of the reporter gene as GUS enzyme activity only in transformed plant cells, i.e. crown gall. However, the formation and growth of galls caused by C58 pIG 121 were much slower than by C58. One month after inoculation with C58 pIG 121, the galls were excised and used to assay for GUS expression using X-Gluc histochemical staining. 79.1% galls or calli from the inoculated shoots that were pretreated with water expressed GUS (Plate 3.10A), while only 60.4% of galls or calli from BTH-treated shoots were GUS-positive. Tissue from the control shoots which were wounded without *A. tumefaciens* inoculation showed no blue coloration (Plate 3.10B).

Table 3.2 Response of *in vitro* rose shoots (var. Madam Isaac Pieriere) 4 days after treatment with or without BTH to subsequent infection by *A. tumefaciens*

BTH (μ M)	No. of tested shoots	% of shoots with galls	Mean diameter of gall (mm)
0	24	65.50 ± 7.93	3.71 ± 0.42
50	24	39.59 ± 3.76	2.33 ± 0.14

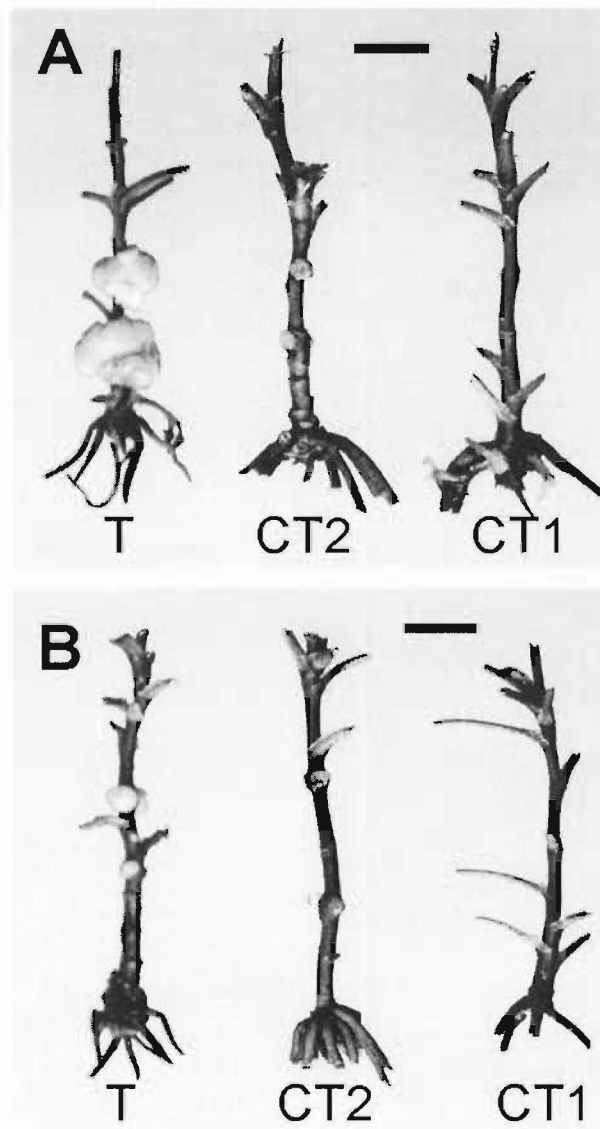


Plate 3.9 Effect of BTH application on gall formation of *in vitro* rose shoots (var. Madam Isaac Pieriere) after 2 weeks inoculation with *A. tumefaciens* C58.

(A) water-treated; (B) BTH-treated (50 μ M). CT1: non-wounded and non-inoculated; CT2: wounded and inoculated with water; T: gall formation on the water-treated or the BTH-treated shoots followed by inoculation with *A. tumefaciens*. The photographs were taken after 2 weeks from inoculation. Scale bars = 5 mm.

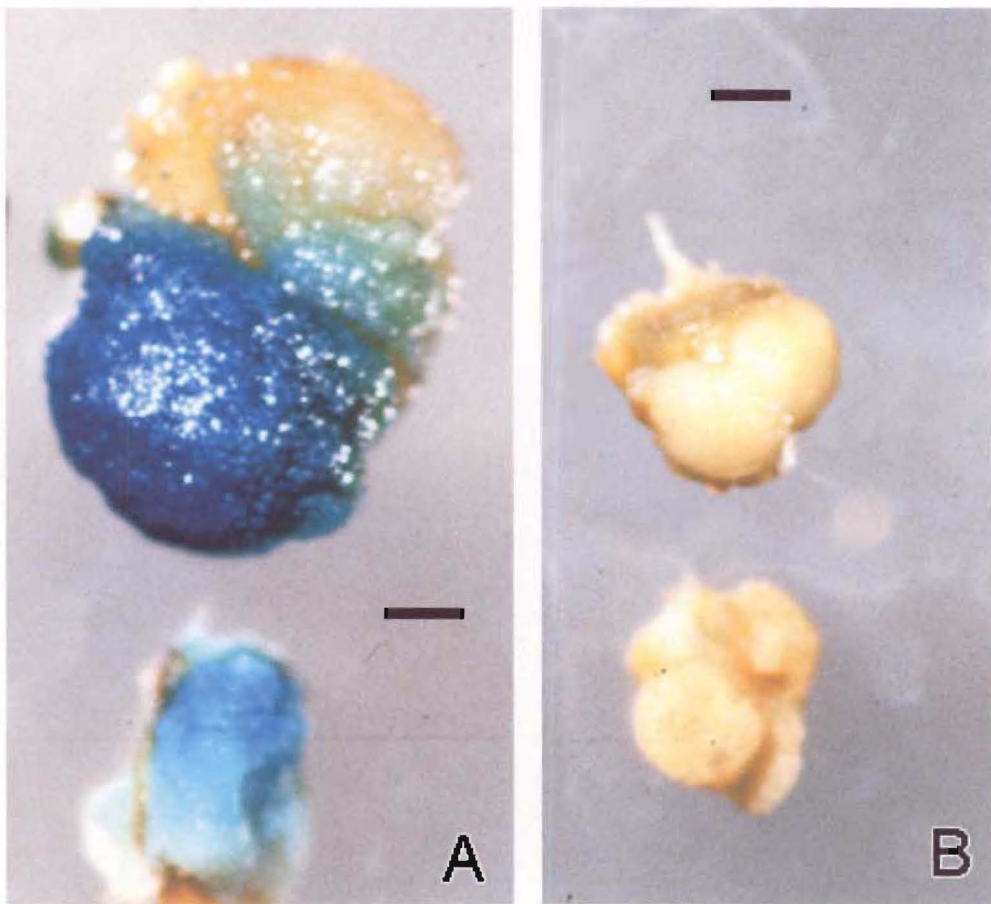


Plate 3.10 Histochemical detection of GUS activity in galls induced by *A. tumefaciens* strain C58 pIG 121.

(A) GUS expression of galls from *in vitro* rose shoots (Madam Isaac Pieriere) first treated with water before inoculation with *A. tumefaciens* strain C58 pIG 121. (B) No Gus activity was detected in calli from wounded shoots. The photographs were taken after 4 weeks from inoculation with the agrobacteria.

3.2 Changes in the patterns of extracellular proteins following BTH treatment

It has been demonstrated that BTH works via activating host plant defence mechanisms rather than a direct mode of action. The BTH-induced resistance is tightly associated with the expression of a set of PR genes in a variety of plant species (Friedrich et al. 1996, Lawton et al. 1996, Siegrist et al. 1997, Inbar et al. 1998, Colson-Hanks and Deverall 2000). Accumulation of PR proteins associated with disease resistance has already been demonstrated, including resistance induced by pathogen infection and chemical treatment (Linthorst 1991, Stintzi et al. 1993, Kessmann et al. 1994, Van Loon 1997). These findings have given rise the hypothesis that PR proteins may play an important role in plant defence response. Since many PR-proteins are localised in the intercellular spaces of leaves (Bol et al. 1990, Linthorst 1991, Stintzi et al. 1993), it would be interesting to investigate the protein changes in the intercellular fluids from rose leaves after BTH treatment to understand the mechanisms by which BTH induce resistance to *D. rosae* and *A. tumefaciens*.

3.2.1 ICF extraction and its efficiency

In order to investigate changes of proteins in the intercellular fluids from BTH-treated and water-treated leaves, a modified ICF extraction procedure was developed based on the method of De Wit and Spikman (1982). Over a period of 7 days, there was no consistent difference in the yield of ICF from leaves treated with BTH or water, varying from 150 µl to 260 µl per gram of leaves.

Five days after treatment with 50 µM BTH or water, the leaves of rose shoots grown *in vitro* were harvested and infiltrated with three different solutions, i.e. 0.1 mM citrate-phosphate buffer pH 2.8, 0.1 mM Tris-HCl buffer pH 8.0, and distilled water. The ICF extracts were then analysed by SDS-PAGE (Plate 3.11). A few bands were detected in ICFs extracted with low pH buffer. However, there were no major qualitative changes in the protein patterns of ICFs extracted with water or high pH buffer. Thus, water was further used as the extract solution in all ICF extracts in this study.

In comparison with silver staining, Coomassie Brilliant Blue staining only revealed the more abundant proteins induced by BTH (Plate 3.12). Silver staining can identify some additional weak bands. The 14, 15, 27, 29, 34 kDa protein was stained poorly with Coomassie Brilliant Blue, but was readily detected by silver staining. The 26, 36 and 54 kDa proteins were intensely stained by both methods. Thus, silver staining was further chosen as the method of staining the proteins on gels in this study.

3.2.2 Determination of ICF contamination

To test if the ICFs were contaminated by intracellular proteins, α -mannosidase was used as a marker for the extent of cytoplasmic leakage/release during the ICF extraction process (Boller and Kende 1979). ICFs were extracted from the leaves of *in vitro* rose shoots after 5 days from the treatment with water, BTH and ethephon. After this, the remaining leaf tissues (minus the ICFs) were homogenised with 0.1 M sodium acetate buffer (pH 5.2) to prepare a cell-free extract. The ICFs and cell extracts were subsequently determined for the activity of α -mannosidase. The data indicated that more than 91% of α -mannosidase activities were detected in the cell extracts, whereas less than 9% was found in the ICFs (Table 3.3). In addition, the ICF extracts were yellowish rather than greenish, indicating that no major breakdown of cells occurred. Therefore, this ICF extraction procedure is an efficient and reliable method for recovering of ICF proteins.

Table 3.3 α -mannosidase activities in intra- and extracellular extracts from leaves of *in vitro* rose shoots (var. Iris Gee)

Treatment	Enzyme activity*		
	Cell extract**	ICF	Percentage of ICF/cell extract
Water	28.79	1.24	4.31
BTH (50 μ M)	29.18	1.26	4.32
Ethephon (10 mM)	33.38	2.98	8.93

* Absorbance units at 405 nm per hour per g of fresh weight at 37 °C.

** The extracts were prepared after the removal of ICF

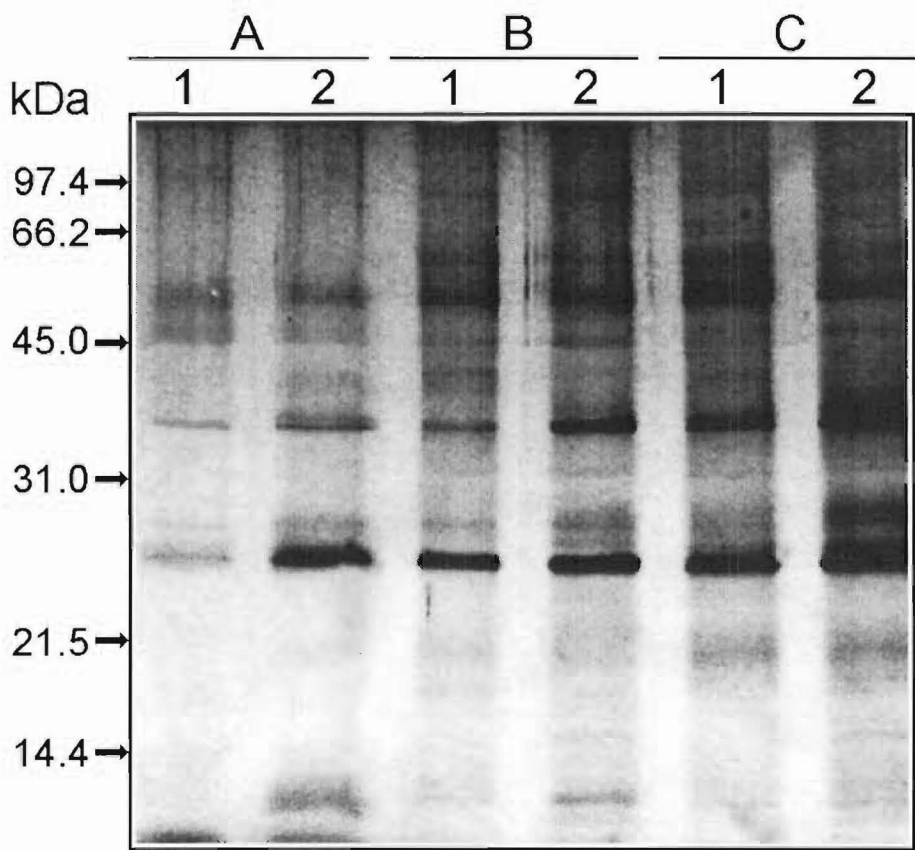


Plate 3.11 SDS-PAGE patterns of extracellular proteins extracted from leaves of *in vitro* rose shoots (var. Iris Gee) with three different buffers.

Whole *in vitro* rose shoots were dipped into either a 50 μ M BTH solution (lane 2) or distilled water (lane 1) for a few seconds under aseptic conditions before they were returned to the shoot growth medium. Five days after the treatments, the leaves were harvested and extracted with: (A) 0.1 mM citrate-phosphate buffer pH 2.8; (B) distilled water; and (C) 0.1 mM Tris-HCl buffer pH 8.0. Each lane was loaded with 5 μ g proteins. The gel was stained with AgNO_3 .

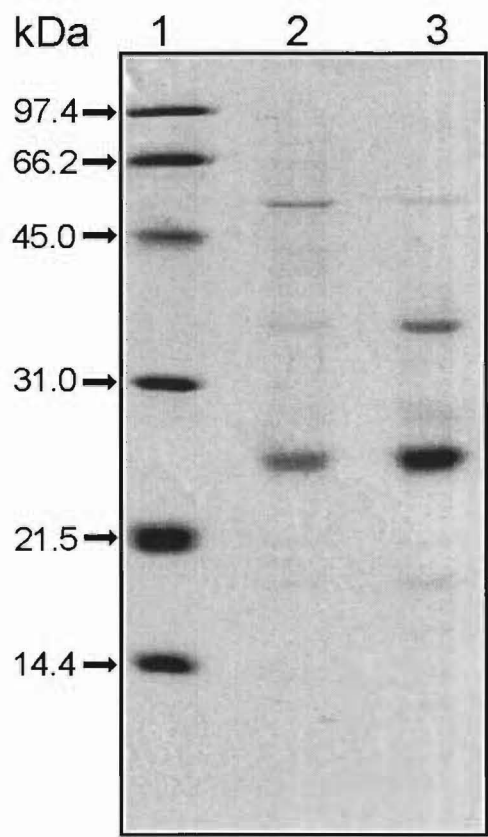


Plate 3.12 Coomassie Brilliant Blue staining of a SDS-PAGE gel showing patterns of extracellular proteins from leaves of *in vitro* rose shoots (var. Iris Gee) following BTH treatment.

Lane 1: marker proteins. Lane 2 and lane 3: the intercellular fluids extracted from the leaves 5 days after treatment with water and 50 μ M BTH, respectively. Each lane was loaded with 20 μ g proteins. The gel was stained with Coomassie Brilliant Blue. The molecular weights of marker proteins are indicated on the left in kDa.

3.2.3 Protein content

The changes in protein content after treatments with 50 μ M BTH or water over 7 days were determined. In the control, protein levels remained constant with slight fluctuations. The protein content dropped in 48 h of growth, probably because of the uptake of water. In BTH-treated leaves, the content of protein markedly increased from day 3 after treatment and continued increasing until day 7 (Figure 3.3).

3.2.4 Analysis by SDS-PAGE of extracellular proteins

3.2.4.1 Effect of different BTH concentrations

The effect of BTH treatments on the patterns of extracellular proteins in rose leaves was examined at day 5 after the treatments. When the extracellular proteins from BTH treatments were compared with those from control (water) after SDS-PAGE and silver staining, some quantitative and qualitative differences were observed (Plate 3.13). Several proteins between 10 and 40 kDa were induced by BTH at concentrations greater than 5 μ M BTH. Some of the bands were newly present and some increased in abundance, particularly at the higher BTH concentrations. No significant differences were found between the treatment with 2.5 mM and 5 mM BTH. Higher than 0.5 mM BTH strongly induced a light yellow band with an approximate molecular weight of 36 kDa, which was also found in senescent leaves. However, a 10 kDa protein induced by 50 μ M BTH was apparently absent in other treatments.

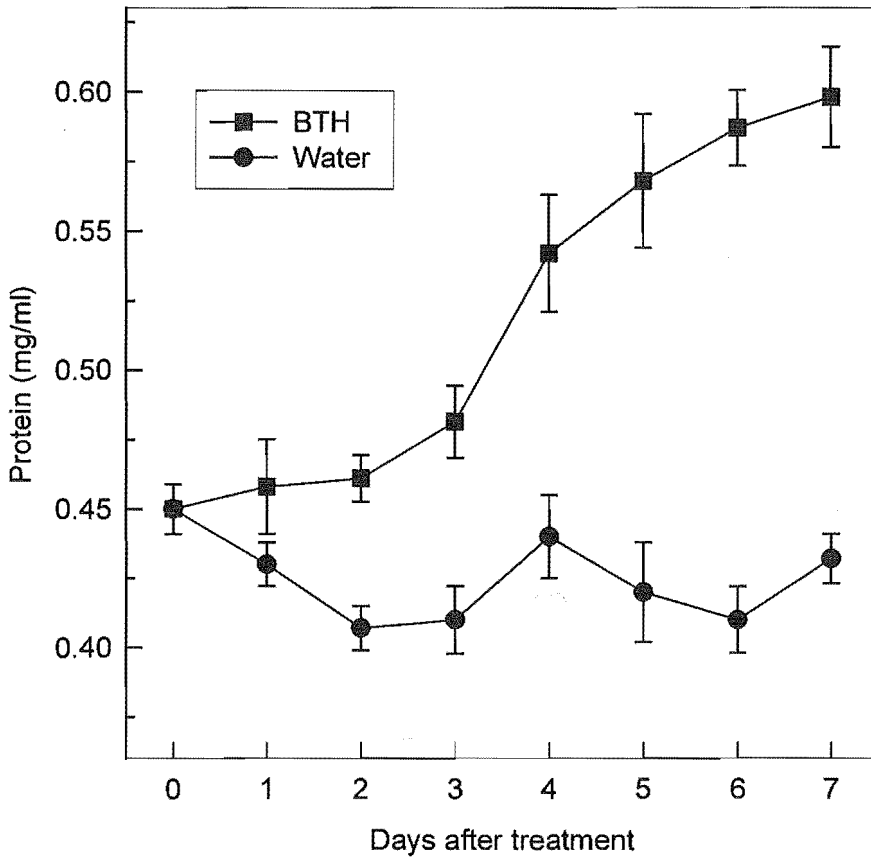


Figure 3.3 Time course of protein content in the intercellular fluids from leaves of *in vitro* rose shoots (var. Iris Gee) after treatment with 50 µM BTH.

Whole *in vitro* rose shoots were dipped into either a 50 µM BTH solution or water for a few seconds under aseptic conditions before they were returned to the shoot growth medium. Protein concentrations in the intercellular fluids extracted from the leaves after treatments over a 7-day period were measured. Each bar represents a mean \pm standard error from three replicates.

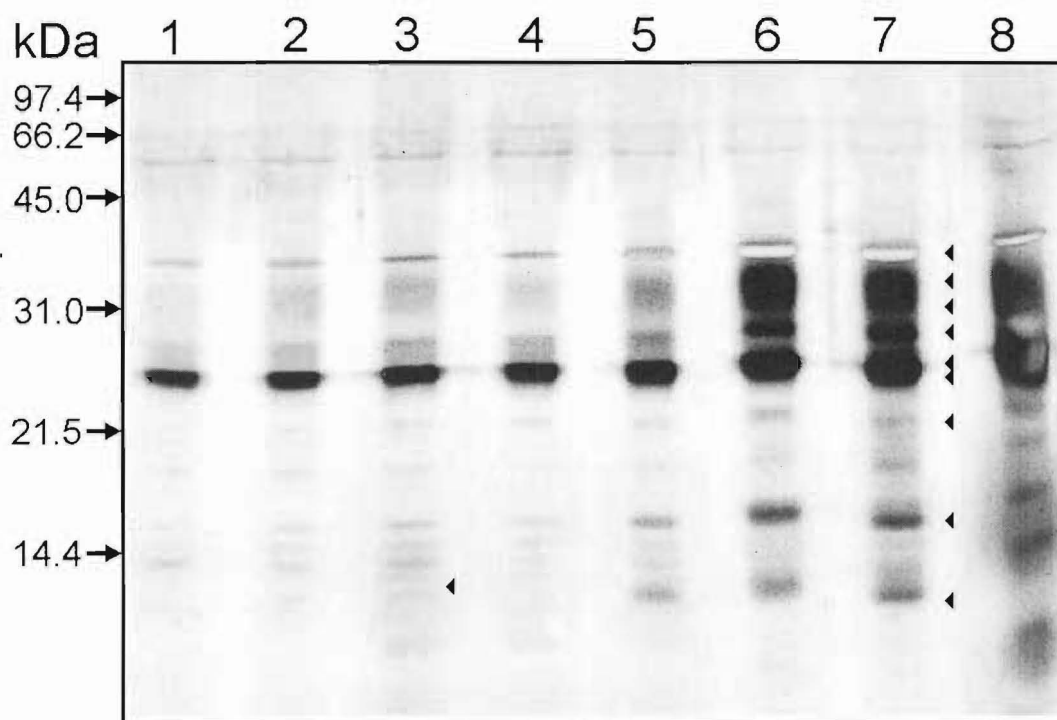


Plate 3.13 SDS-PAGE patterns of extracellular proteins extracted from leaves of *in vitro* rose shoots (var. Iris Gee) after treatment with different concentrations of BTH.

BTH was applied by dipping the whole *in vitro* rose shoots into the chemical at different concentrations or water for a few seconds before returning them to shoot growth medium. ICFs were extracted at day 5 after the various treatments, separated by SDS-PAGE in a standard protein electrophoresis unit, and stained with AgNO_3 . Lane 1 (water); Lane 2 (5 μM BTH); Lane 3 (50 μM BTH); Lane 4 (50 μM BTH[†]); Lane 5 (500 μM BTH); Lane 6 (2.5 mM BTH); Lane 7 (5 mM BTH); and Lane 8 (senescent leaves). Each lane contained 10 μg proteins. Arrows indicate the proteins that were induced by BTH treatments. The molecular weights of marker proteins are indicated on the left in kDa.

3.2.4.2 Time course of BTH-induced extracellular proteins

The changing patterns of extracellular proteins from the leaves of *in vitro* rose shoots after treatment with 50 μ M BTH over a 7-day period were shown in Plate 3.14. One and two days after the BTH treatment, there were apparently no marked changes in extracellular proteins. Most of the induced proteins started to accumulate after three days from the start of the treatment. At day 5, accumulation of the induced proteins was clearly evident.

In comparison with the leaves treated with 50 μ M BTH, the protein accumulation patterns in the intercellular fluids were different when treated with 5 mM BTH over the period of 7 days (Plate 3.15). The 36 kDa protein, a light yellow band, started to accumulate from day 3 after this BTH treatment.

3.2.4.3 Extracellular protein changes by addition of BTH to the culture medium

Different concentrations of BTH were added into culture medium to test if BTH added into the culture medium could still effectively induce the accumulation of the extracellular proteins as induced by the dipping method. The ICFs from the leaves of different treatments were prepared as before and analysed by SDS-PAGE (Plate 3.16). The results showed that two application methods of BTH induced similar changes of protein patterns (compare Plate 3.16 with Plate 3.13). However, the intensities of some induced proteins were appreciably lower when BTH was applied to the medium than by dipping shoots into the BTH solution at the same concentration. This is probably because it was difficult for the shoots without roots to take up the BTH from the medium or the chemical was not distributed quickly throughout the shoots.

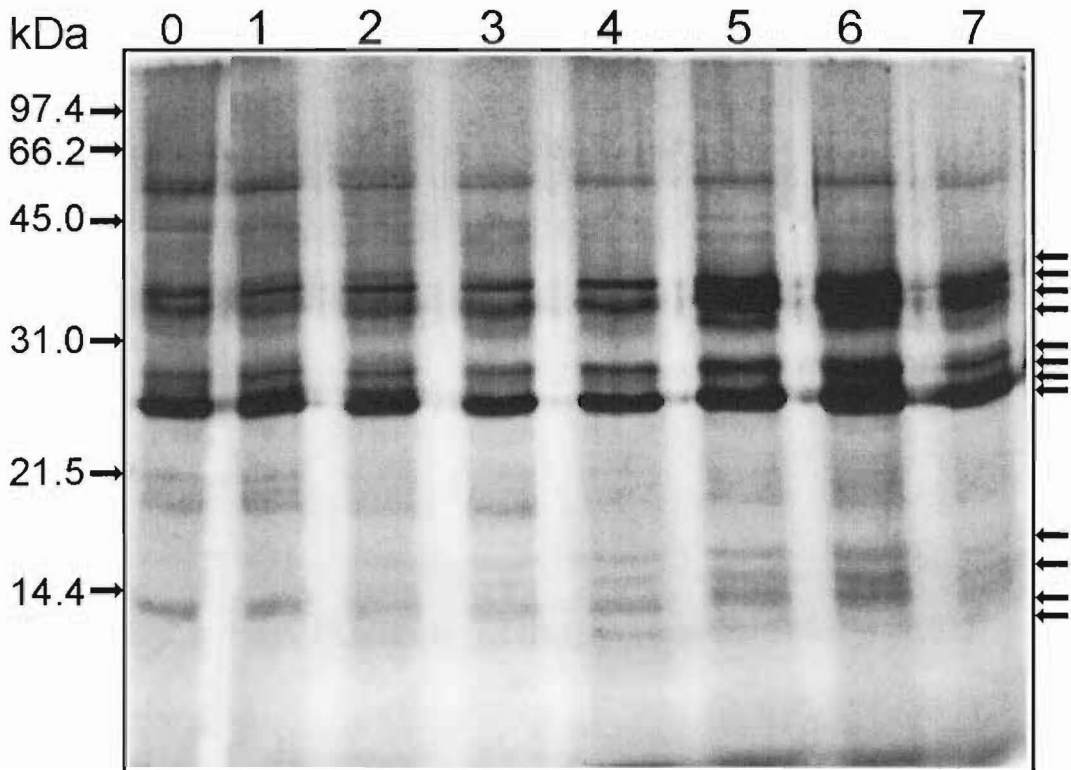


Plate 3.14 Accumulation of extracellular proteins from leaves of *in vitro* rose shoots (var. Iris Gee) following treatment with 50 μ M BTH over 7 days.

BTH was applied by dipping whole *in vitro* rose shoots into a 50 μ M solution of the chemical for a few seconds. After treatment, the shoots were put back to culture medium. The intercellular fluids of leaves were extracted after the BTH treatment at day 0, 1, 2, 3, 4, 5, 6, and 7 (lanes 0 to 7, respectively), and separated by SDS-PAGE in a Mini-PROTEAN II electrophoresis cell. Each lane was loaded 5 μ g proteins. The gel was stained with AgNO_3 . Arrows indicate the proteins were strongly induced by 50 μ M BTH treatment. The molecular weights of marker proteins are indicated at the left in kDa.

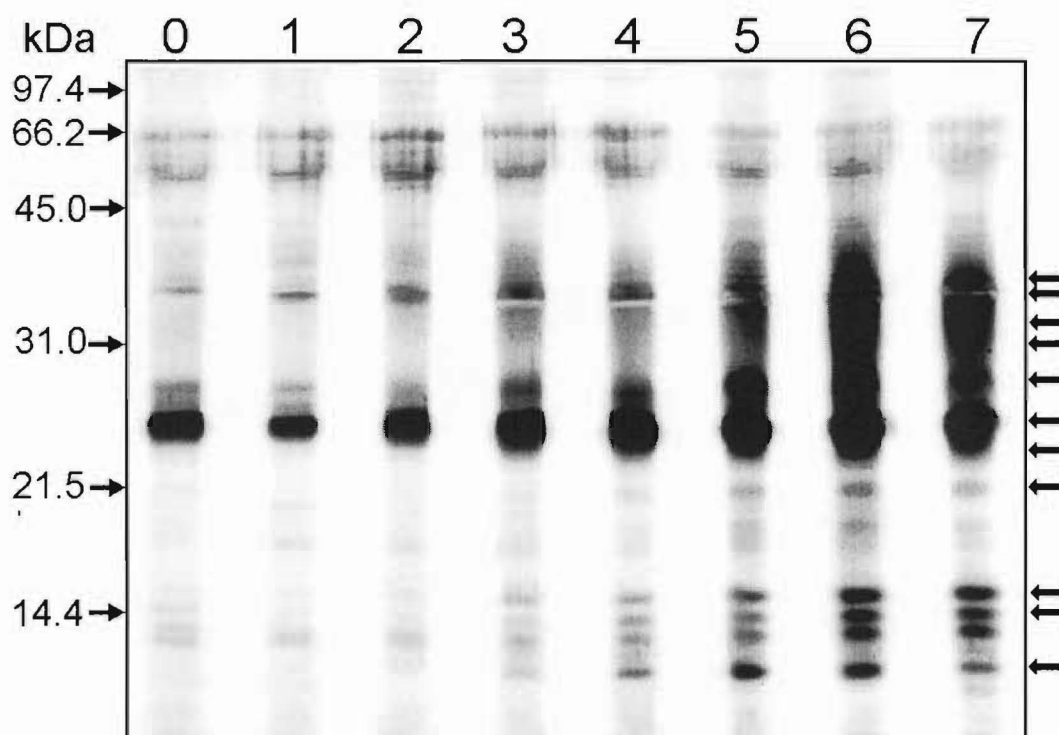


Plate 3.15 Accumulation of extracellular proteins from leaves of *in vitro* rose shoots (var. Iris Gee) following treatment with 5 mM BTH over 7 days.

BTH was applied by dipping whole *in vitro* rose shoots into a 5 mM solution of the chemical for a few seconds. After treatment, the shoots were put back to culture medium. The intercellular fluids of leaves were extracted after the BTH treatment at day 0, 1, 2, 3, 4, 5, 6, and 7 (lanes 0 to 7, respectively), and separated by SDS-PAGE in a standard protein electrophoresis unit. Each lane was loaded 10 μ g proteins. The gel was stained with AgNO_3 . Arrows on the right-hand side indicated the proteins were strongly induced by 5 mM BTH treatment. The molecular weights of marker proteins are indicated at the left in kDa.

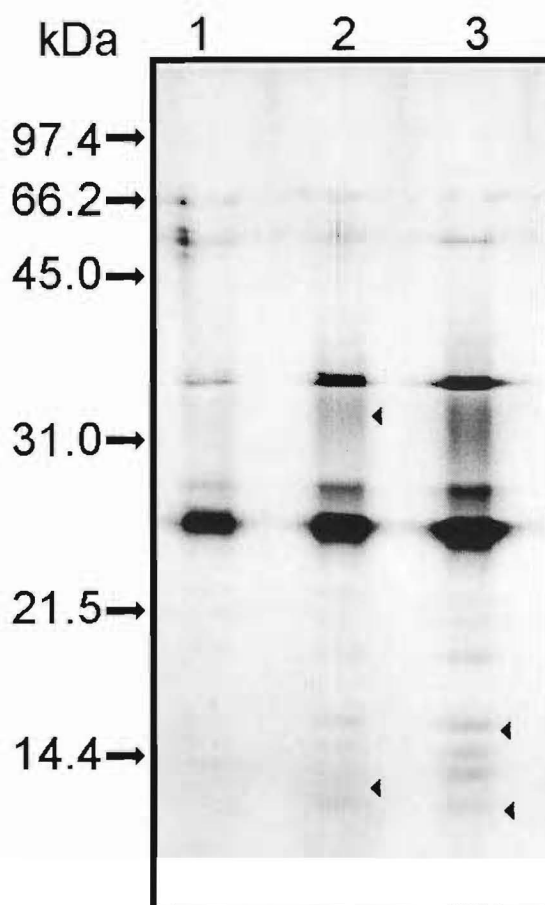


Plate 3.16 SDS-PAGE patterns of extracellular proteins from leaves of *in vitro* rose shoots (var. Iris Gee) grown in the shoot culture medium supplemented with 3 different concentrations of BTH.

BTH was applied by adding it into the shoot culture medium. Lane 1: ICF extracted from control, Lane 2 and 3: ICF extracted from the leaves of shoots growing in shoot culture medium containing 50 μ M and 500 μ M BTH, respectively. The ICFs were analysed by SDS-PAGE and the gel was stained with AgNO_3 . Each lane contained 10 μ g proteins. Arrows indicate the intensities of the induced proteins by BTH were appreciably lower in comparison with the method by dipping shoots into the BTH solution at the same concentration. The molecular weights of marker proteins are indicated at the left in kDa.

3.2.5 Analysis by 2D-PAGE of extracellular proteins

SDS-PAGE analysis of ICF extracts revealed some differences in the protein patterns of BTH-treated vs. untreated leaves. However, in order to determine the protein profile more precisely in relation to the molecular weights and pIs of the proteins, the ICFs were further analysed by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). The leaves of *in vitro* rose shoots were used as the experimental material and the time courses of extracellular protein changes of both water-treated and 50 μ M BTH-treated leaves at day 0, 1, 3, 5, and 7 after the start of the experiment were compared.

Most of the proteins present in the intercellular fluids of control leaves were also found in those of the BTH-treated leaves (Plates 3.17-3.20). Compared with the control, fourteen proteins were induced or increased in abundance during 7 days after the BTH treatment. The levels of 7 proteins, i.e. No. 8, 10, 12, 13, 14, 15 and 16 with apparent molecular weights of 25.5, 27, 28, 33, 34, 36 and 37 kDa, respectively, were enhanced by the BTH treatment, while the other 7 proteins, i.e. No. 1, 2, 3, 4, 11, 17, and 18 with apparent molecular weights of 14, 14, 15, 15, 27.5, 38, and 28 kDa, respectively, were only detectable on the gels with the extracts from the BTH-treated leaves. The induced proteins No. 12, 15 and 16 increased in abundance starting after 1 day from the BTH treatment and reaching a maximum at day 7, while most of induced proteins were found to accumulate after 3 days from the BTH treatment (Plates 3.17-3.20 and Table 3.4).

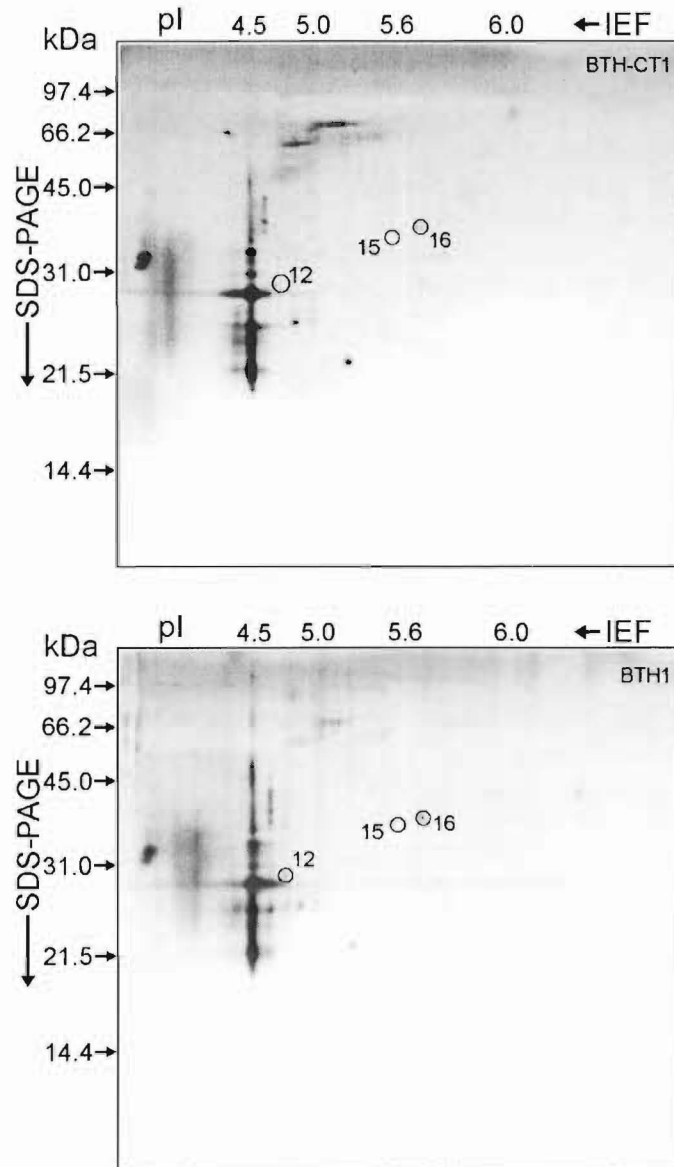


Plate 3.17 Two-dimensional gel electrophoresis of extracellular proteins from leaves of *in vitro* rose shoots (var. Iris Gee) 1 day after treatment with 50 μ M BTH or water.

Upper: water (BTH-CT1); Bottom: BTH treatment (BTH1). Twenty micrograms of proteins were separated by 2D-PAGE followed by silver staining. Numbers at the left margin indicate the molecular weights of marker proteins in kDa. The open circles mark proteins that increased in their levels at day 1 after the BTH treatment.

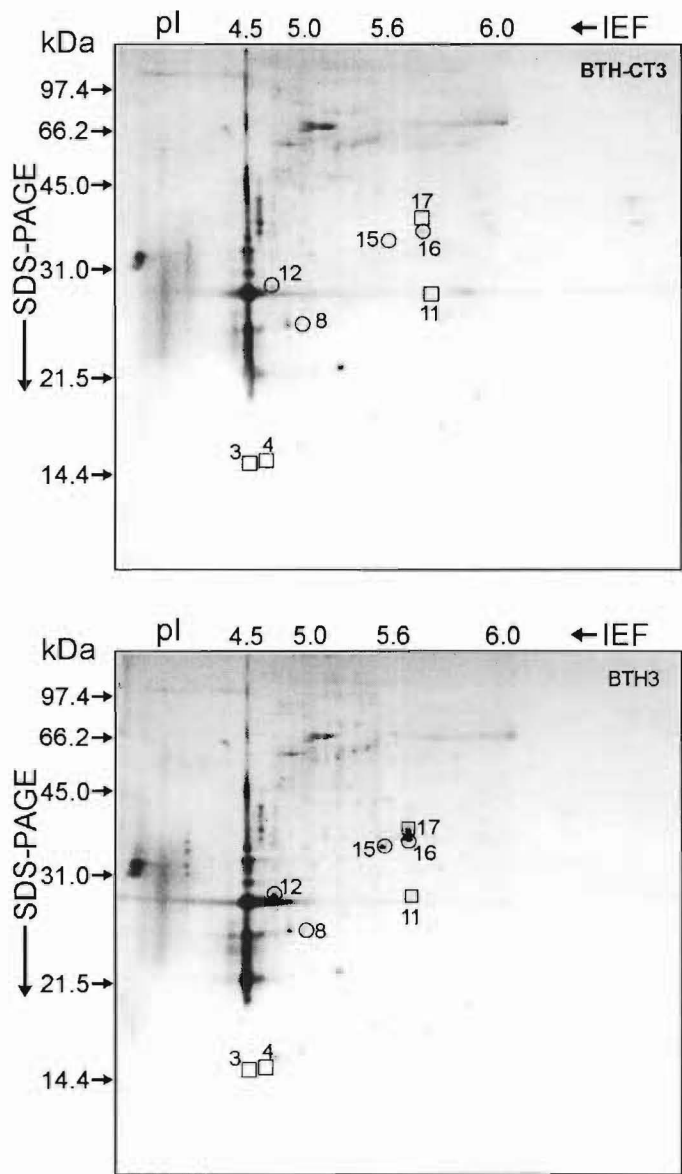


Plate 3.18 Two-dimensional gel electrophoresis of extracellular proteins from leaves of *in vitro* rose shoots (var. Iris Gee) 3 days after treatment with 50 μ M BTH or water.

Upper: water (BTH-CT3); Bottom: BTH treatment (BTH3). Twenty micrograms of proteins were separated by 2D-PAGE followed by silver staining. Numbers at the left margin indicate the molecular weights of marker proteins in kDa. The open squares indicate the proteins only detected in the BTH treatment; the open circles mark proteins that increased in their levels at day 3 after the BTH treatment.

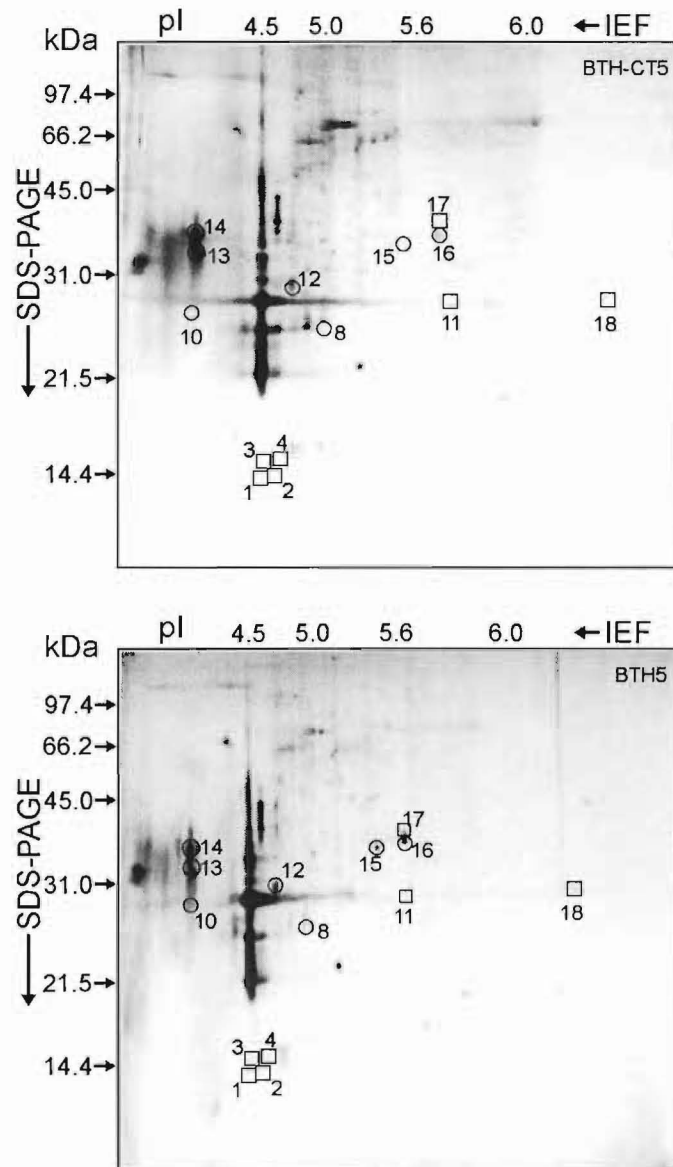


Plate 3.19 Two-dimensional gel electrophoresis of extracellular proteins from leaves of *in vitro* rose shoots (var. Iris Gee) 5 days after treatment with 50 μ M BTH or water.

Upper: water (BTH-CT5); Bottom; BTH treatment (BTH5). Twenty micrograms of proteins were separated by 2D-PAGE followed by silver staining. Numbers at the left margin indicate the molecular weights of marker proteins in kDa. The open squares indicate the proteins only detected in the BTH treatment; the open circles mark proteins that increased in their levels at day 5 after the BTH treatment.

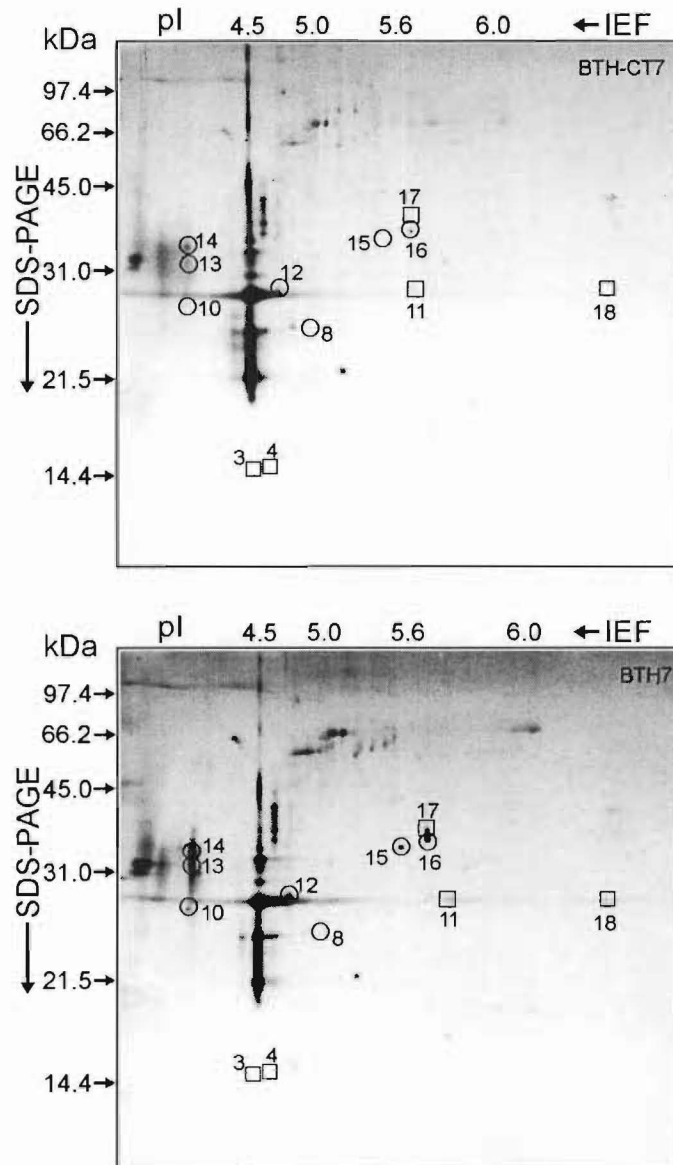


Plate 3.20 Two-dimensional gel electrophoresis of extracellular proteins from leaves of *in vitro* rose shoots (var. Iris Gee) 7 days after treatment with 50 μ M BTH or water.

Upper: water (BTH-CT7); Bottom; BTH treatment (BTH7). Twenty micrograms of proteins were separated by 2D-PAGE followed by silver staining. Numbers at the left margin indicate the molecular weights of marker proteins in kDa. The open squares indicate the proteins only detected in the BTH treatment; the open circles mark proteins that increased in their levels at day 7 after the BTH treatment.

Table 3.4 A summary of the changes of the extracellular proteins from leaves of *in vitro* rose shoots (var. Iris Gee) after 50 μ M BTH treatment over 7 days

Proteins			BTH treatments (days)			
Number	MW(kDa)	pI	1	3	5	7
1	14	4.5	-	-	+	-
2	14	4.6	-	-	+	-
3	15	4.5	-	+	+	+
4	15	4.6	-	+	+	+
8	25.5	4.9	+	++	++	+
10	27	4.0	+	+	++	++
11	27.5	5.75	-	+	+	+
12	28	4.7	++	++	++	++
13	33	4.0	+	+	++	++
14	34	4.0	+	+	++	++
15	36	5.5	++	++	++	++
16	37	5.7	++	++	++	++
17	38	5.7	-	+	+	+
18	28	>7	-	-	+	+

(-) indicate the protein that was not detected in the treatment at a given time.

(+) indicate the protein that was detected in the treatment at a given time.

(++) indicate the protein that was detected and became more abundant in some treatments.

3.2.6 Identification of the proteins induced by BTH

3.2.6.1 The induced proteins were identified by polyclonal antibodies

In an effort to identify novel proteins induced by BTH in rose, the intercellular fluid from the leaves of *in vitro* rose shoots (var. Iris Gee) after 5 days from the treatment with 50 μ M BTH was used to produce polyclonal antibodies.

Western blot analysis revealed that the polyclonal antibodies recognised a number of antigens with molecular weights higher than 24 kDa in the ICFs of both water-treated and BTH-treated leaves (Plate 3.21A). To better demonstrate the specific BTH-induced proteins, the original polyclonal antibodies were repeatedly adsorbed by the ICF extract from the water-treated leaves. Thus, most of the antibodies reacting with the antigens in the ICF of water-treated leaves were excluded from the adsorbed polyclonal antibodies. It was clearly shown that the adsorbed serum detected eight bands (arrows) in BTH-treated leaves, while only three bands were present in control (Plate 3.21B), suggesting BTH activated a set of genes in rose as demonstrated in other plant species (Friedrich et al. 1996, Görlach et al. 1996, Lawton et al. 1996). The result also indicated that the adsorption procedure was basically efficient although three major bands were still found in both the extracts of BTH-treated and water-treated leaves. The two bands indicated by open arrows were also detected on rose ICF extracts against preimmune serum (data not shown), which were due to non-specific binding of primary antibodies.

In order to determine if the PR proteins induced by blackspot infection are also induced by BTH treatment, polyclonal antibodies raised against the ICF from the infected leaves 7 days after inoculation with *D. rosae* were prepared. The antiserum detected a large number of bands that were present in the ICFs of both water-treated and BTH-treated leaves (Plate 3.22A). Repeating the adsorption as above, the resulting adsorbed serum reacted with twelve antigens in the BTH-treated leaves. In contrast, few proteins were detected in the water-treated leaves (Plate 3.22B). The proteins with low molecular weights (< 24 kDa) were undetectable on the western blots probed with the polyclonal antibodies, unless the gels were overloaded with the ICFs (data not shown). This is probably because the antigens smaller than 24 kDa were relatively scarce in the intercellular fluids compared to other proteins.

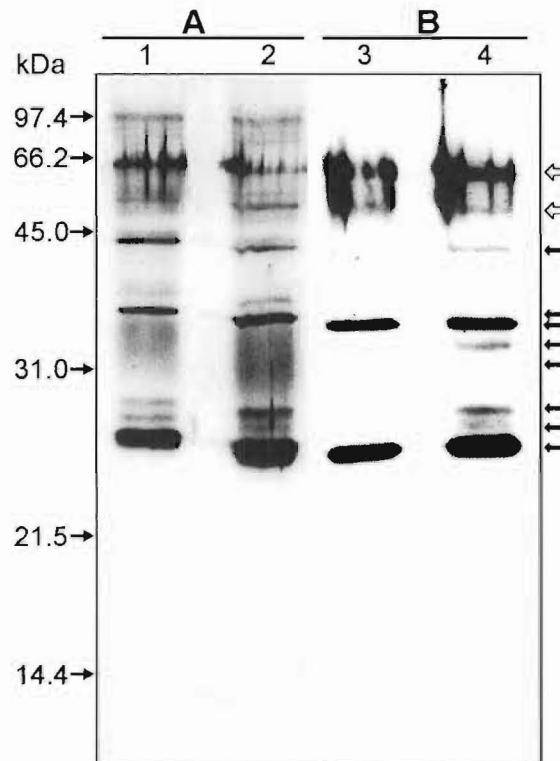


Plate 3.21 Western blots showing the proteins detected by polyclonal antibodies raised against the ICF extracted from BTH-treated leaves of *in vitro* rose shoots (var. Iris Gee).

ICF extracts were separated by SDS-PAGE and then the proteins were transferred onto a PVDF membrane. Lanes 1 and 3: the ICF from the water-treated leaves; Lanes 2 and 4: the ICF from the BTH-treated (50 μ M) leaves. (A) The blot was incubated with polyclonal antibodies raised against the ICF from the leaves of *in vitro* rose shoots after 5 days from the treatment with 50 μ M BTH. (B) The blot was incubated with the polyclonal antibodies after adsorption against the ICF of the water-treated leaves. Arrows indicate the proteins that were detected in the ICF extract from the BTH-treated leaves by the adsorbed polyclonal antibodies. Open arrows indicate the bands that were also detected on rose ICF extracts against preimmune serum. The molecular weights of marker proteins stained with Coomassie Brilliant Blue R-250 are indicated on the left in kDa.

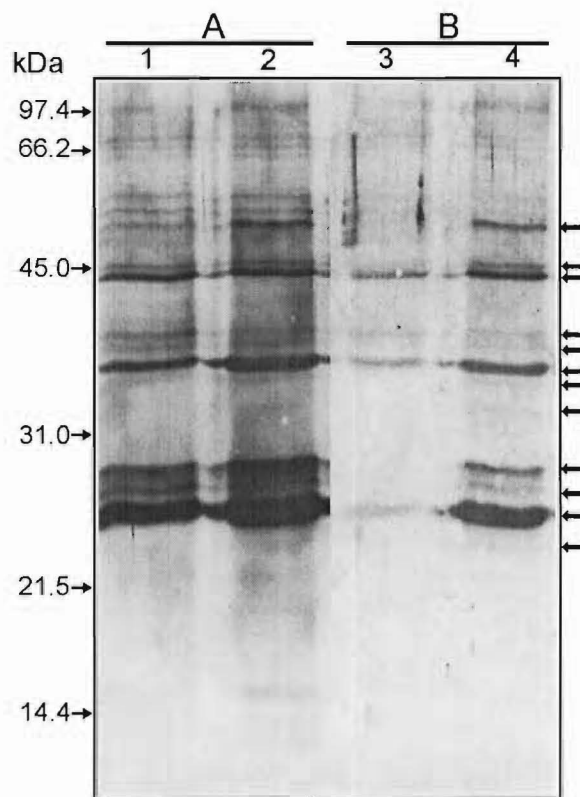


Plate 3.22 Western blots showing the proteins detected by polyclonal antibodies raised against the ICF extracted from blackspot-infected leaves of *in vitro* rose shoots (var. Iris Gee).

ICF extracts were separated by SDS-PAGE and then the proteins were transferred onto a PVDF membrane. Lanes 1 and 3: the ICF from the water-treated leaves; Lanes 2 and 4: the ICF from the BTH-treated (50 μ M) leaves. (A) The blot was incubated with polyclonal antibodies raised against the ICF from the leaves of *in vitro* rose shoots after 7 days from the inoculation with *D. rosae*. (B) The blot was incubated with the polyclonal antibodies after adsorption against the ICF of the water-treated leaves. Arrows indicate the proteins that were appreciably detected in the ICF extract from the BTH-treated leaves by the adsorbed polyclonal antibodies. The molecular weights of marker proteins stained with Coomassie Brilliant Blue R-250 are indicated on the left in kDa.

3.2.6.2 Western blots probed with antisera against tobacco PR proteins

The antisera raised against the four major classes of tobacco PR proteins, i.e. PR-1c protein (PR-1 family) (Jamet and Fritig 1986), PR-N protein (PR-2 family) (Kauffmann et al. 1987), PR-Q protein (PR-3 family) (Legrand et al. 1987), PR-S protein (PR-5 family) (Kauffmann et al. 1990), which were kindly provided by Dr. Kauffmann, were used to detect the kinetics of accumulation of the related PR proteins in the intercellular fluids from the leaves of *in vitro* rose shoots after treatment with 50 μ M BTH. The ICFs extracted from control and BTH-treated leaves at various times after treatments were separated by SDS-PAGE and the proteins were then transferred onto nitrocellulose membranes and used for immunoblot analysis.

The serological relationship between rose BTH-induced proteins and tobacco PR proteins was demonstrated by western blot analyses (Plate 3.23 and 3.24). It was showed that the accumulation of the PR-1 protein with apparent molecular weight of 15 kDa occurred after 2 days from the onset of the BTH treatment and reached a maximum level at day 5 in the ICFs of BTH-treated leaves (Plate 3.23). In contrast, it was undetectable in the ICF of the water-treated leaves. The other two rose proteins of approximately 27 and 38 kDa also reacted with the tobacco anti-PR-1c, particularly the former one. But, there was no apparent difference in their expression between the BTH treatment and water control. The nature or function of these proteins, which might be associated with PR-1 or due to unspecific binding of the antibody, remains to be clarified.

Three bands of PR-2 proteins could be detected in the ICF extracts from BTH-treated leaves (Plate 3.23). The molecular weights of the bands were about 26, 36 and 37 kDa, respectively. There was a dramatic increase in the levels of PR-2 proteins at 3 days after BTH treatment, but not in the leaves of control shoots. However, in a preliminary experiment, low levels of PR-2 and PR-3 counterparts were detected when the gels were overloaded.

Three PR-3 proteins against tobacco anti-PR-Q were detected (Plate 3.24). The proteins with molecular weights of approximate 25 and 33 kDa, respectively, which were found in low levels in water-treated leaves, slightly increased with times after BTH treatment, while the protein of approximate 37.5 kDa, which is not detected in control, accumulated only in the ICFs extracted from BTH-treated leaves starting after 4 days from the BTH treatment and remaining at this level from day 4 to 7.

Two bands with apparent molecular weights of about 25.5 and 27.5 kDa in the ICFs of the BTH-treated leaves were recognised by the tobacco anti-PR-S (Plate 3.24). They started to accumulate at day 3 after BTH treatment. However, they were apparently absent in the ICFs of the water-treated leaves.

3.2.6.3 Two-dimensional immunoblotting to identify PR proteins

In order to further characterise PR proteins, extracellular proteins extracted from the leaves of *in vitro* rose shoots (var. Iris Gee) after 5 days from treatment with 50 μ M BTH were separated on 2D-PAGE gels before they were transferred onto PVDF membranes. The membranes were first stained with Coomassie Brilliant Blue to mark the spots. Immunoblots of 2D gels were analysed using antisera raised against tobacco PR-1c, PR-N, PR-Q and PR-S proteins. Anti-PR-1c failed to detect PR-1 proteins from BTH-treated leaves. Probably, not enough rose proteins were loaded on the 2D gel for visualisation of the spots. Further experiment was not attempted, as only limited amount of the tobacco PR-1c antiserum was available. Three PR-2 spots were detected with the tobacco anti-PR-N (Plate 3.25A). Four spots were recognised as PR-3 proteins (Plate 3.25B) and two spots as PR-5 proteins (Plate 3.25C). The spots corresponding to the proteins of silver stained 2D-PAGE were matched (Plate 3.25D). The properties of the rose PR proteins in relation to the four major classes of tobacco PR proteins were summarised in Table 3.5 based on the western blot analyses in this study.

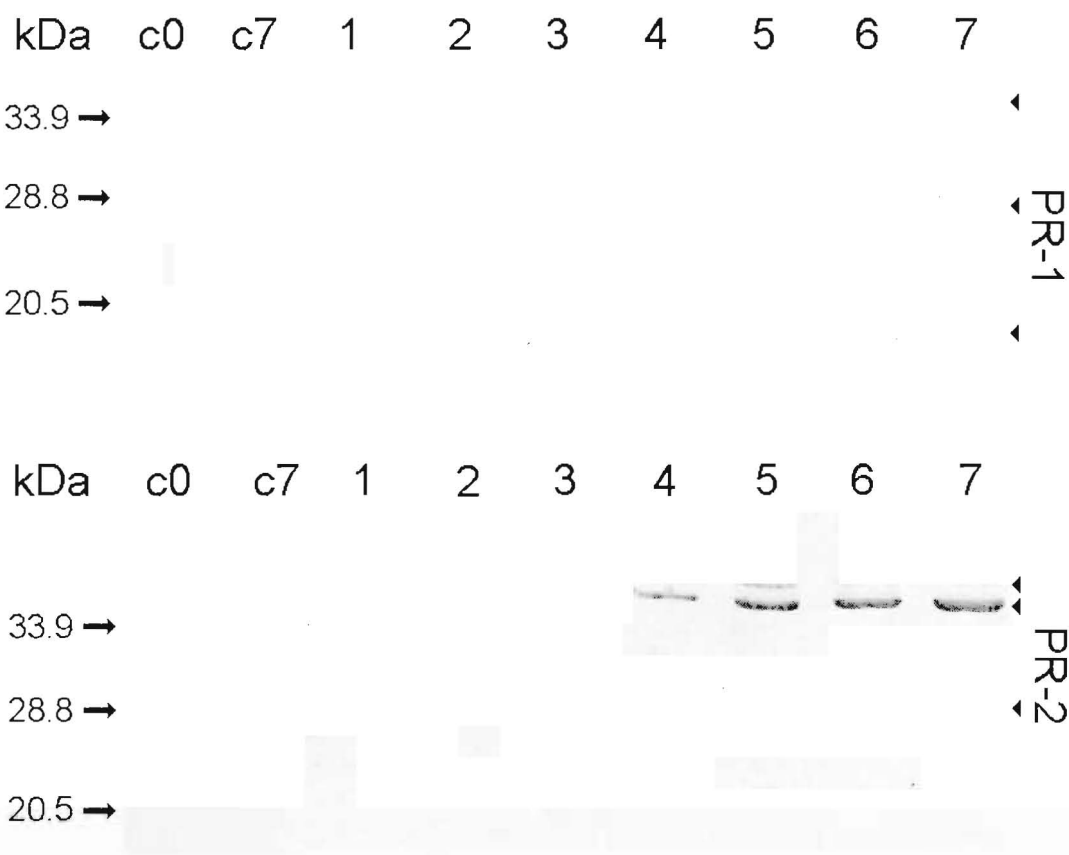


Plate 3.23 Western blots showing the accumulation of PR-1 and PR-2 proteins in the ICFs from leaves of *in vitro* rose shoots (var. Iris Gee) after treatment with 50 μ M BTH.

Whole *in vitro* rose shoots (var. Irish Gee) were treated with water (Lanes C0 and C7) or BTH (Lanes 1 to 7). The ICFs extracted from the leaves at various times from the start of the treatment with water or BTH (1-7 d) were separated on 15% SDS-PAGE gels and the proteins were subsequently transferred onto nitrocellulose membranes. The blots were probed with antisera raised against tobacco PR-1c protein (PR-1 family) and PR-N protein (PR-2 family). The molecular weights of prestained marker proteins are indicated on the left in kDa.

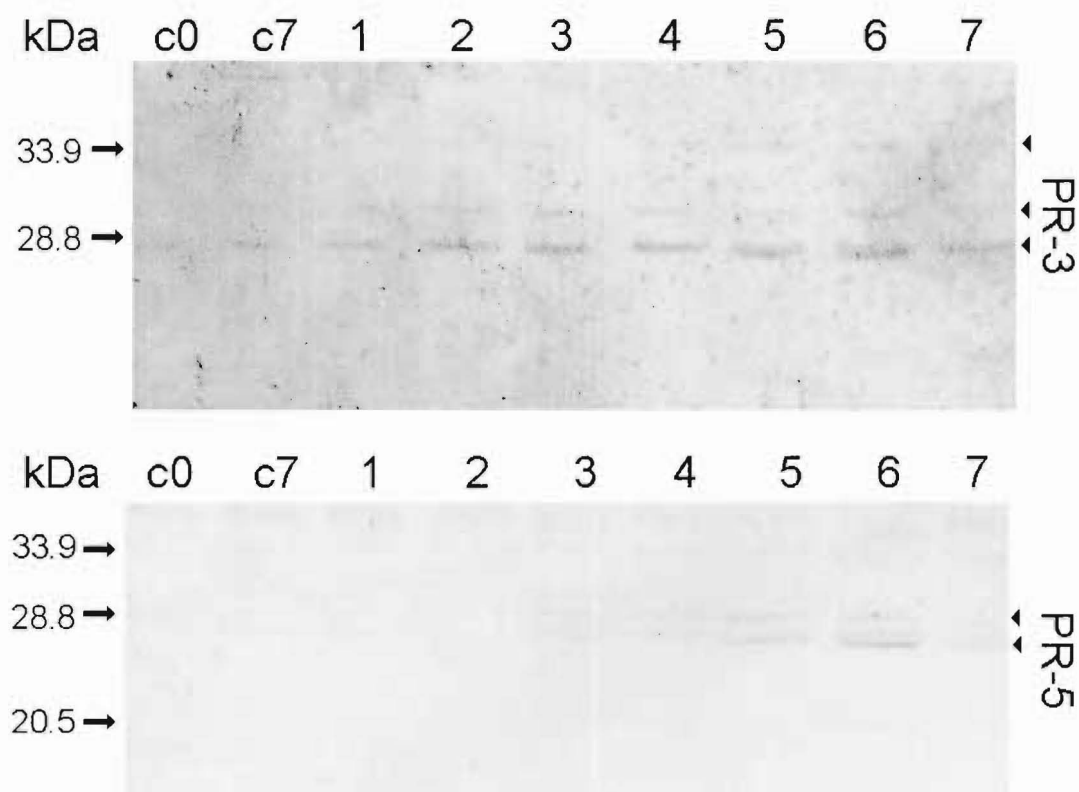


Plate 3.24 Western blots showing the accumulation of PR-3 and PR-5 proteins in the ICFs from leaves of *in vitro* rose shoots (var. Iris Gee) after treatment with 50 μ M BTH.

Whole *in vitro* rose shoots (var. Iris Gee) were treated with water (Lanes C0 and C7) or BTH (Lanes 1 to 7). The ICFs extracted from the leaves at various times from the start of the treatment with water or BTH (1-7 d) were separated on 15% SDS-PAGE gels and the proteins were subsequently transferred onto nitrocellulose membranes. The blots were probed with antisera raised against tobacco PR-Q protein (PR-3 family) and PR-S protein (PR-5 family). The molecular weights of prestained marker proteins are indicated on the left in kDa.

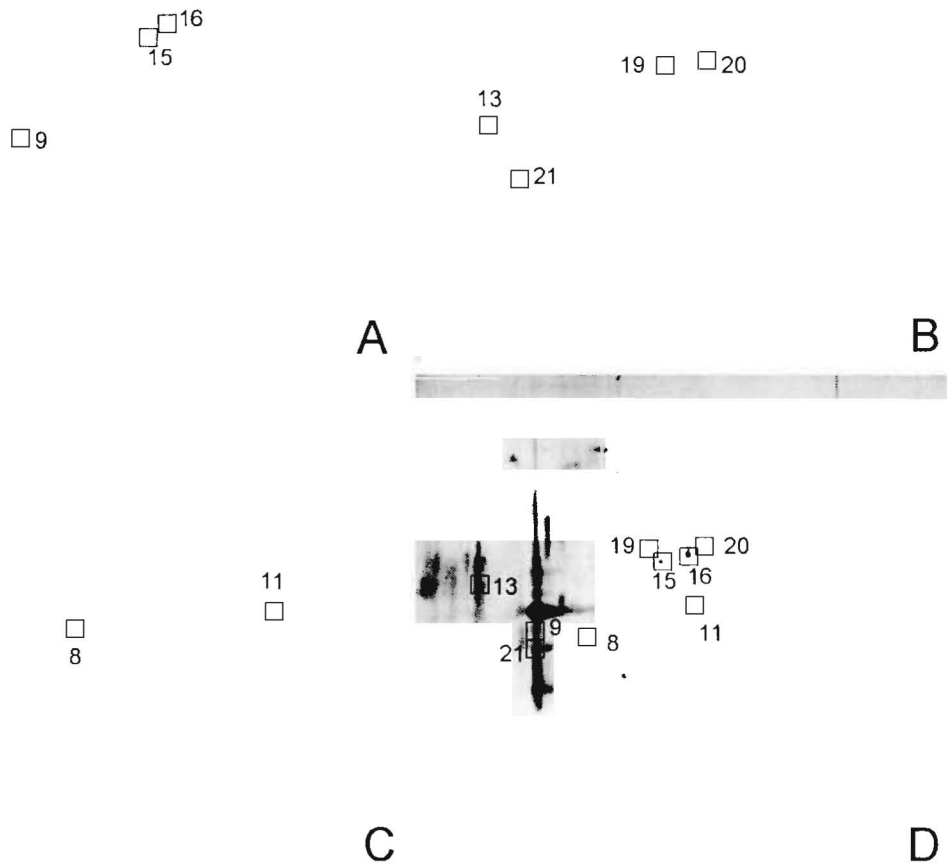


Plate 3.25 Two-dimensional immunoblots of the ICF from leaves of *in vitro* rose shoots after 5 days from treatment with 50 μ M BTH.

The ICFs extracted from the leaves after 5 days from the treatment with 50 μ M BTH were subjected to 2D-PAGE, and then the proteins were transferred onto PVDF membranes. The membranes were first stained with Coomassie Brilliant Blue to mark the spots. After this, the blots were probed with antisera raised against tobacco PR-N protein (PR-2 family) (A), PR-Q protein (PR-3 family) (B), and PR-S protein (PR-5 family) (C), and silver stained gel (D).

Table 3.5 A summary of the characteristics of the BTH-induced PR proteins in the ICFs of rose leaves studied with 4 classes of tobacco PR proteins

Classes of tobacco PR proteins	No. of serological isoforms	Molecular weights (kDa)	Isoelectric points (pI)
PR-1	3	15	-
		27	-
		38	-
PR-2 (β -1,3-glucanases)	3	26	4.5
		36	5.5
		37	5.7
PR-3 (Chitinases)	4	25	4.5
		33	4.0
		37.5	5.4 & 5.8
PR-5 (thaumatin-like)	2	25.5	4.9
		27.5	5.75

— : the proteins that were not detected on 2-D immunoblots.

3.2.7 Purification and microsequencing of some prominent BTH-induced proteins

In an effort to characterise the BTH-induced proteins, the intercellular fluid extracted from the leaves of *in vitro* rose shoots after 5 days from treatment with 50 μ M BTH was separated by 2D-PAGE and blotted onto a PVDF membrane. The preliminary experiments demonstrated that some spots of interest could not be detected clearly after staining with Coomassie Brilliant Blue. One reason could be that the proteins had poor resolution as a larger amount of proteins was loaded on the gel in this experiment than that in the previous experiments with silver staining. The other possible reason is that the proteins were not fixed well on the Coomassie Blue stained PVDF membranes. There were some marked differences in resolution when proteins were fixed with or without acetic acid in the staining and destaining solutions. In order to avoid blockage of the amino terminus, acetic acid was not added in the staining and destaining solutions following the instructions of the PVDF membrane supplier (*BIO-RAD*) as the ultimate purpose was for protein sequencing from the PVDF blots. In order to improve the poor resolution of proteins, a greater glass tube with 2.5 mm internal diameter was used for the first dimension IEF and thicker gels for the second dimension SDS-PAGE.

Three spots, the most strongly induced proteins that accumulated in BTH-treated leaves, were cut off from the membrane and sent to Department of Biochemistry, University of Otago, New Zealand for their N-terminal sequence analyses. However, the obtained results were inconclusive (see Appendix 7) because the levels of proteins in the spots were too low to enable to give convincing sequences. According to the suggestion of sequence analyst, the spots of No. 15 and 16 collected from four or six electroblots were probably required to obtain the N-terminal sequences. Thus, further experiments were not carried out as larger amounts of ICF need to be extracted from the leaves of hundreds of *in vitro* rose shoots.

3.3 Changes in the patterns of extracellular PR proteins following infection with *D. rosae*

PR proteins have been identified in at least nine plant families, including more than 30 species (Van Loon et al. 1994, Mohamed and Sehgal 1997). It has been so extensively studied as the accumulation of PR proteins is tightly correlated with hypersensitive response and systemic acquired resistance which are efficient plant defence responses against pathogen attack in nature. Besides, most groups of PR proteins possess antifungal activity *in vitro* and *in vivo* (Linthorst 1991, Stintzi et al. 1993, Ryals et al. 1996, Van Loon 1997). The inducible PR proteins are mostly acidic extracellular proteins which have been suggested to be the first line of plant defence to a challenging pathogen (Van Loon 1997). Therefore, extracellular proteins of leaves have been the subject of extensive research. However, up to now, there is no report on the occurrence of PR proteins in rose. In order to manipulate the natural defence capabilities of rose via biotechnology, it is an important prerequisite to investigate the occurrence, characteristics and regulation of PR proteins, particularly, those accumulating in the intercellular spaces of rose leaves. In this part, the changing patterns of PR proteins in the intercellular fluids of rose leaves after inoculation with *D. rosae* were investigated.

3.3.1 Accumulation of extracellular PR proteins

About 150-250 µl intercellular fluid (ICF) could be obtained from one gram leaf tissue of *in vitro* rose shoots. As compared with control, the ICF of infected leaves was more yellowish, indicating that they might contain more phenolic substances (Parent and Asselin 1984).

The intercellular fluids extracted from leaves at various times after inoculation were subjected to analysis by SDS-PAGE. As shown on Plate 3.26, at least ten proteins showing qualitative and quantitative changes were found in the intercellular spaces of infected leaves compared with control (healthy leaves at day 0 and those newly expanded/upper uninfected leaves). Several proteins started to accumulate at day 3 after inoculation. By day 7, when the blackspot disease symptoms were fully developed on leaves, the induced PR proteins became more prominent in the inoculated leaves. The 24 and 29 kDa proteins were presented abundantly in infected leaves, whereas these bands were absent from the control (Plate 3.26). With the defoliation of leaves, the intensity of some bands was reduced at 10 days after inoculation.

Isoelectric focusing (IEF) gel electrophoresis followed by silver staining indicated that most of the induced proteins in the ICF from rose leaves infected with *D. rosae* had acidic isoelectric points (Plate 3.27).

Changes in the patterns of extracellular protein accumulation after 7 days from inoculation with or without *D. rosae* were further analysed by high resolution two-dimensional gel electrophoresis (2D-PAGE). Since the proteins accumulated in the ICF of infected leaves are mainly acidic, special attention was paid to the region from pH 4-8 in the IEF gel of the first dimension electrophoresis. About 120 spots were identified by silver staining following 2D-PAGE (Plate 3.28). Most of the extracellular proteins were found in the vicinity of pI 4.5. Eight proteins (open squares) were newly made and eight proteins (open circles) increased in abundance in infected leaves after 7 days from inoculation. With the aid of 2D-gel marker proteins included in the samples, the molecular weights and isoelectric points of these proteins were estimated (Plate 3.28 and Table 3.6).

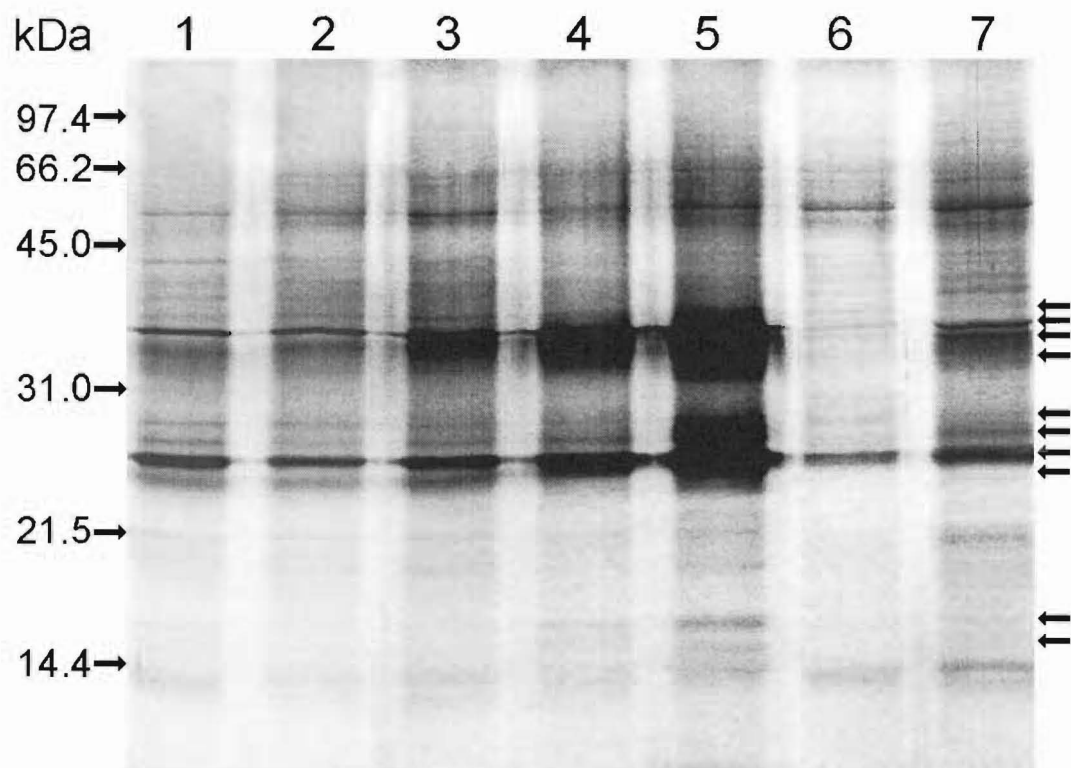


Plate 3.26 The accumulation of PR proteins in the intercellular spaces of rose leaves following inoculation with *D. rosae*.

In vitro rose shoots (var. Iris Gee) were inoculated with *D. rosae* by dipping them into a conidial suspension. The ICFs extracted from the leaves were collected at day 0 (lane 1), day 1 (lane 2), day 3 (lane 3), day 5 (lane 4), and day 7 (lane 5) after inoculation with *D. rosae*. The newly expanded/upper uninfected leaves were also collected from water-inoculated control (lane 6) and from shoots inoculated with the pathogen 7 days after inoculation (lane 7). The extracts (5 μ g proteins in each lane) were separated by SDS-PAGE and stained with AgNO_3 . Arrows on the right-hand side indicate the proteins that became more abundant after inoculation with *D. rosae*. The molecular weights of marker proteins are indicated at the left in kDa. ICFs were prepared and analysed at least twice. Similar results were obtained in the different analyses.

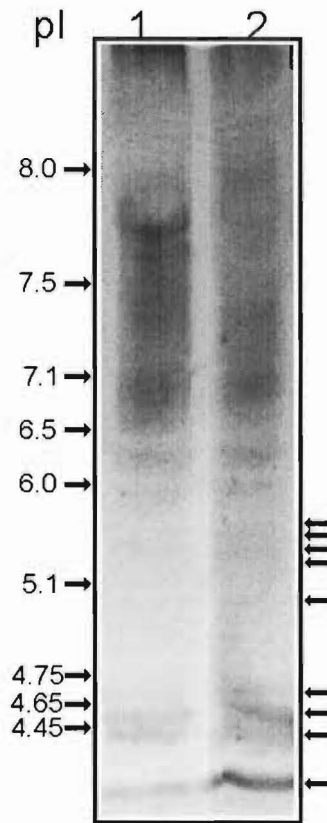


Plate 3.27 Isoelectric focusing gel electrophoresis of extracellular proteins from leaves of *in vitro* rose shoots (var. Iris Gee) inoculated with *D. rosae*.

The ICF extracts from water-inoculated (lane 1) and blackspot-infected (lane 2) rose leaves harvested after 7 days from the inoculation were separated on an isoelectric focusing (IEF) gel (pH 3-10). The gel was silver stained. Arrows on the right-hand side indicate the proteins that became more abundant after inoculation with *D. rosae*. The standard pI marker proteins are indicated at the left.

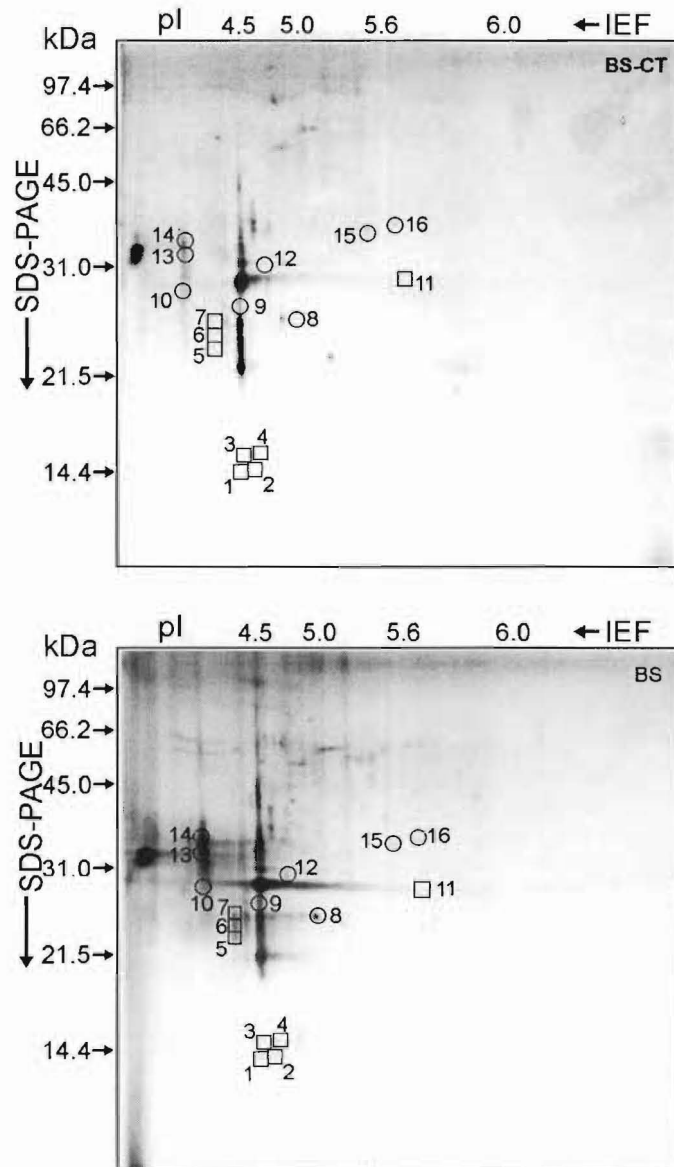


Plate 3.28 Two-dimensional gel electrophoresis of extracellular proteins from leaves of *in vitro* rose shoots (var. Iris Gee) inoculated with *D. rosae*.

Extracellular proteins were extracted from the leaves without inoculation (BS-CT) and from the leaves 7 days after inoculation with *D. rosae* (BS). Twenty micrograms of proteins were separated by 2D-PAGE followed by silver staining. The open squares indicate the proteins that were not detectable in the control gel (BS-CT); the open circles mark an increase in abundance of the proteins after inoculation with *D. rosae*. The molecular weights of marker proteins are indicated at the left in kDa.

Table 3.6 Characteristics of extracellular PR proteins from the leaves of *in vitro* rose shoots (var. Iris Gee) following inoculation with *D. rosae* for 7 days

Number*	Molecular weight (kDa)	Isoelectric point (pI)
1	14	4.5
2	14	4.6
3	15	4.5
4	15	4.6
5	23	4.3
6	24.5	4.3
7	25.5	4.3
8	25.5	4.9
9	26	4.5
10	27	4.0
11	27.5	5.75
12	28	4.7
13	33	4.0
14	34	4.0
15	36	5.5
16	37	5.7

* The numbers here identify the proteins that were indicated on the BS gel in Plate 3.28.

3.3.2 Identification of four major classes of PR proteins accumulating in the intercellular spaces of rose leaves

Western blots of extracellular PR proteins from the leaves of *in vitro* rose shoots at various times after inoculation with *D. rosae* were probed with antisera raised against the four major classes of tobacco PR proteins, i.e. PR-1c, PR-N, PR-Q and PR-S proteins.

Three proteins in the ICFs of blackspot-infected rose leaves were recognised by the tobacco anti-PR-1c, indicating that these proteins are serologically related to those of tobacco (Plate 3.29). One band with an approximate molecular weight of 15 kDa, which is similar to that of tobacco PR-1 proteins, was detected at a later stage of infection, from day 5 to 7 after inoculation with *D. rosae*. The other two bands with molecular weights of approximately 27 and 38 kDa, respectively, were present in the ICFs of both healthy and infected leaves.

Antiserum raised against tobacco PR-N cross-reacted with three low-molecular weight proteins in the ICFs of rose infected leaves (Plate 3.29). The approximate molecular weights of these proteins are 26, 36 and 37 kDa, respectively. The levels of 26 and 36 kDa proteins, which were present at a much reduced level in the healthy leaves, were increased after one day from inoculation. One additional band about 37 kDa was found in infected leaves at day 3 to 7 after inoculation with *D. rosae*, coinciding with the appearance of visible black spots.

Western blot analysis revealed that three proteins in the ICFs of rose infected leaves cross-reacted with the tobacco anti-PR-Q (Plate 3.30). They had apparent molecular weights of 25, 33 and 37.5 kDa, respectively, and were weakly detected in the healthy leaves. After infection with *D. rosae* for 3 days, their levels started to

increase, correlating with the appearance of visible smaller symptoms on inoculated leaves. With the further development of disease symptoms, these bands were more easily detectable.

Two proteins with molecular weights of 25.5 and 27.5 kDa, respectively, cross-reacted with the antiserum raised against tobacco PR-S protein (Plate 3.30). They were detected at very low levels in the ICF of healthy leaves. However, the intensity of the two bands was enhanced at later stages (day 5 to 7) after infection by *D. rosae*.

3.3.3 Do extracellular PR proteins accumulate systemically following infection with *D. rosae*?

In order to investigate the possibility that infection with *D. rosae* may induce the systemic accumulation of PR proteins in noninfected tissue, the newly expanded/upper uninfected leaves were harvested at day 7 from both water- and *D. rosae*-inoculated shoots.

Immunoblot analyses (Plate 3.29 and 3.30) indicated that PR-2 (26 and 36 kDa) and PR-3 (25, 33 and 37.5 kDa) proteins all appeared to accumulate systemically at higher levels in the ICF from the newly expanded and uninfected leaves of *D. rosae*-inoculated shoots, whereas PR-5 (25.5 and 27.5 kDa) proteins were weakly induced. In the group of PR-1 proteins, the protein (15 kDa) was detected only in the infected leaves, but not in the newly expanded/uninfected leaves from the *D. rosae*-inoculated shoots at day 7. In addition, it was shown by silver staining following SDS-PAGE that the proteins (14, 15, 24 and 37 kDa), which were accumulated abundantly in infected leaves, were also undetectable in the newly expanded and upper uninfected leaves 7 days after inoculation with *D. rosae* (Plate 3.26).

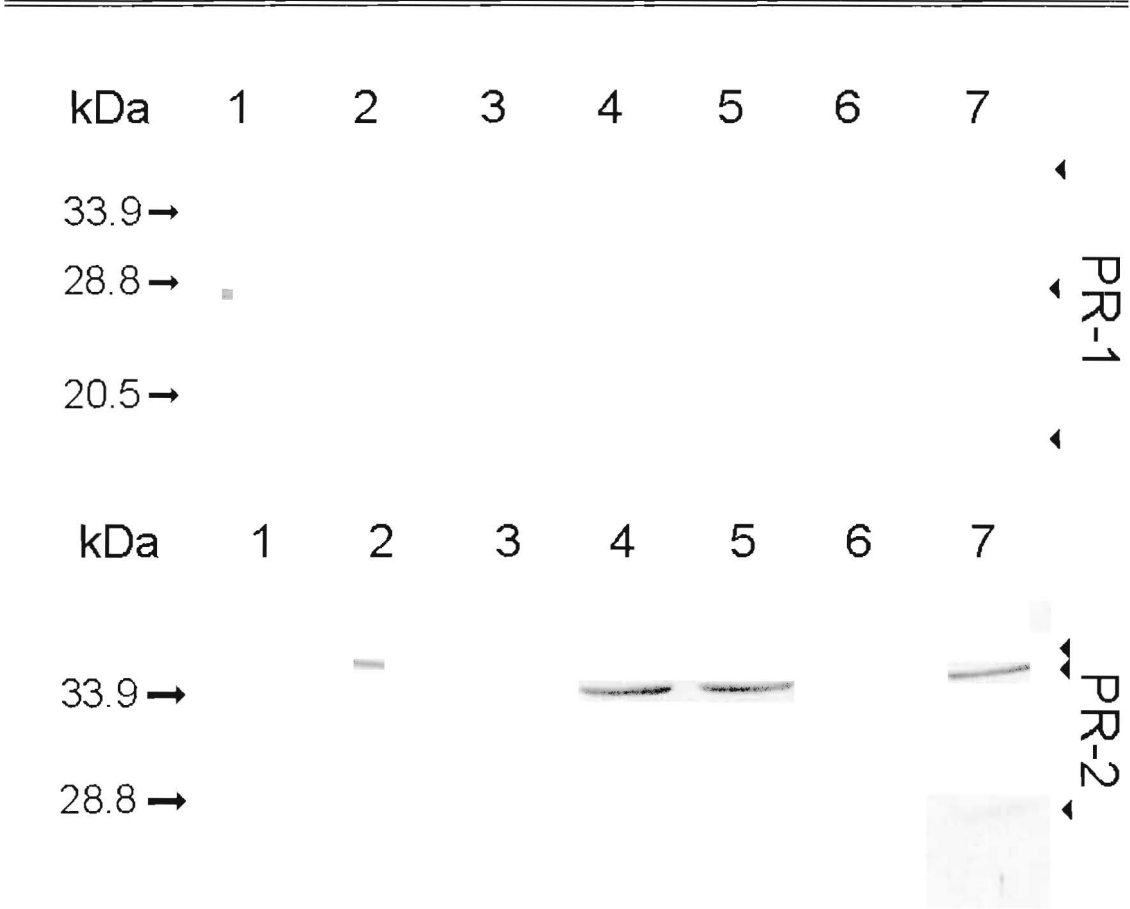


Plate 3.29 Western blots showing the accumulation of PR-1 and PR-2 proteins in the ICFs from leaves of *in vitro* rose shoots (var. Iris Gee) following inoculation with *D. rosae*.

Leaves of rose shoots grown *in vitro* were harvested at day 0 (lane 1), day 1 (lane 2), day 3 (lane 3), day 5 (lane 4), and day 7 (lane 5) after inoculation with *D. rosae*. The newly expanded/upper uninfected leaves appearing since the *in vitro* shoots were inoculated with *D. rosae* (lane 7) as well as those from the water-inoculated control (lane 6) were also collected at day 7. The intercellular fluids were extracted from the leaves and separated by SDS-PAGE. The proteins were subsequently transferred onto nitrocellulose membranes. The blots were probed with antisera raised against tobacco PR-1c protein (PR-1 family) and PR-N protein (PR-2 family). Each lane contained 30 µg proteins for PR-1 and 10 µg proteins for PR-2 protein analysis. The molecular weights of prestained marker proteins are indicated on the left in kDa.

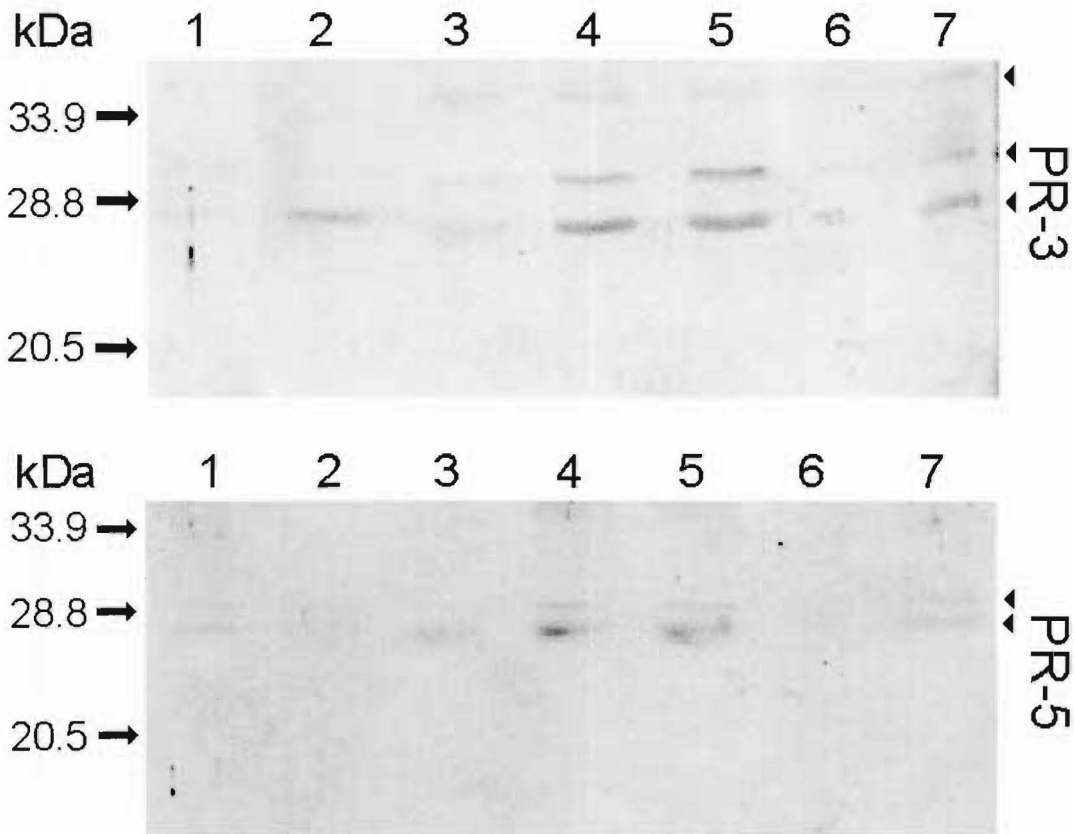


Plate 3.30 Western blots showing the accumulation of PR-3 and PR-5 proteins in the ICFs from leaves of *in vitro* rose shoots (var. Iris Gee) following inoculation with *D. rosae*.

Leaves of rose shoots grown *in vitro* were harvested at day 0 (lane 1), day 1 (lane 2), day 3 (lane 3), day 5 (lane 4), and day 7 (lane 5) after inoculation with *D. rosae*. The newly expanded/upper uninfected leaves appearing since the *in vitro* shoots were inoculated with *D. rosae* (lane 7) as well as those from the water-inoculated control (lane 6) were also collected at day 7. The intercellular fluids were extracted from the leaves and separated by SDS-PAGE. The proteins were subsequently transferred onto nitrocellulose membranes. The blots were probed with antisera raised against tobacco PR-Q protein (PR-3 family) and PR-S protein (PR-5 family). Each lane contained 10 μ g of proteins. The molecular weights of prestained marker proteins are indicated on the left in kDa.

3.4 The effect of BTH pretreatment on the expression of extracellular PR proteins upon challenge inoculation with *D. rosae*

The experiments described here so far demonstrated that both BTH treatment and infection of *D. rosae*, when studied separately, induced the expression and accumulation of the four major classes of extracellular PR proteins through western-blot analyses. However, it has been reported that BTH is not only able to induce PR proteins directly but can also condition the expression of some PR proteins upon pathogen infection in several plant species (Kästner et al. 1998, Katz et al. 1998). In light of these results, it was of great interest to investigate whether BTH is also able to potentiate the expression of PR proteins in leaves of rose shoots after challenge inoculation with *D. rosae*.

Levels of the four major classes of PR protein were generally higher in BTH-pretreated than in water-pretreated leaves followed by inoculation with *D. rosae*, although the pattern of expression was similar (compare Plate 3.31 and 3.32 with Plate 3.29 and 3.30). Particularly, the induction of the PR-1 protein with an apparent molecular weight of 15 kDa and the PR-2 proteins with apparent molecular weights of 36 and 37 kDa was more pronounced (Plate 3.31). These results may indicate either a combined effect of BTH treatment and blackspot infection or a conditioning effect of BTH application on the increased expression of these genes.

SDS-PAGE analysis of extracellular proteins also revealed that the PR proteins induced by *D. rosae* seemed to be present at higher levels from the infected leaves pretreated with BTH (Plate 3.33) than with water (Plate 3.26).

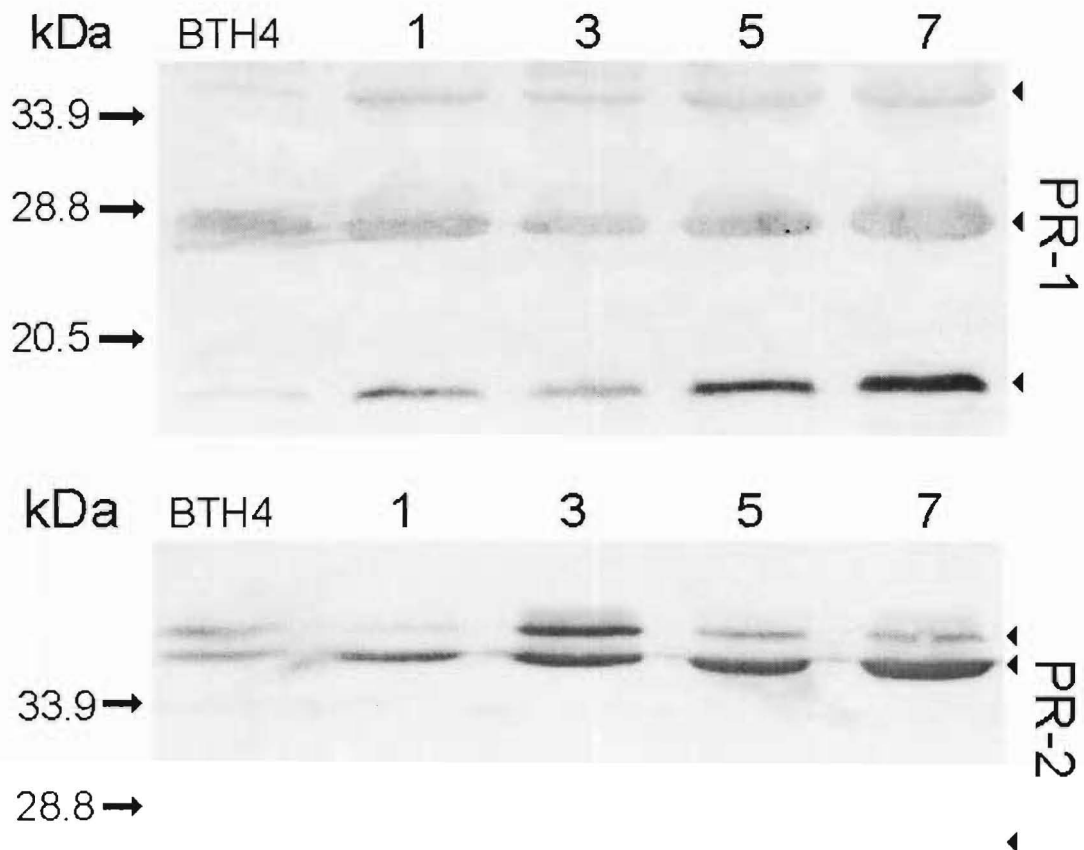


Plate 3.31 The expression of extracellular PR-1 and PR-2 proteins in BTH-pretreated leaves upon inoculation with *D. rosae*.

Whole *in vitro* rose shoots (var. Iris Gee) were first treated with 50 μ M BTH and 4 days later challenge-inoculated with *D. rosae*. Intercellular fluids were extracted at day 0 (lane BTH4), 1, 3, 5, and 7 following inoculation as indicated. After SDS-PAGE, the proteins were subsequently transferred onto nitrocellulose membranes. The blots were probed with antisera raised against tobacco PR-1c protein (PR-1 family) and PR-N protein (PR-2 family). Each lane contained 30 μ g proteins for PR-1 and 10 μ g proteins for PR-2 protein analysis. The molecular weights of prestained marker proteins are indicated on the left in kDa.

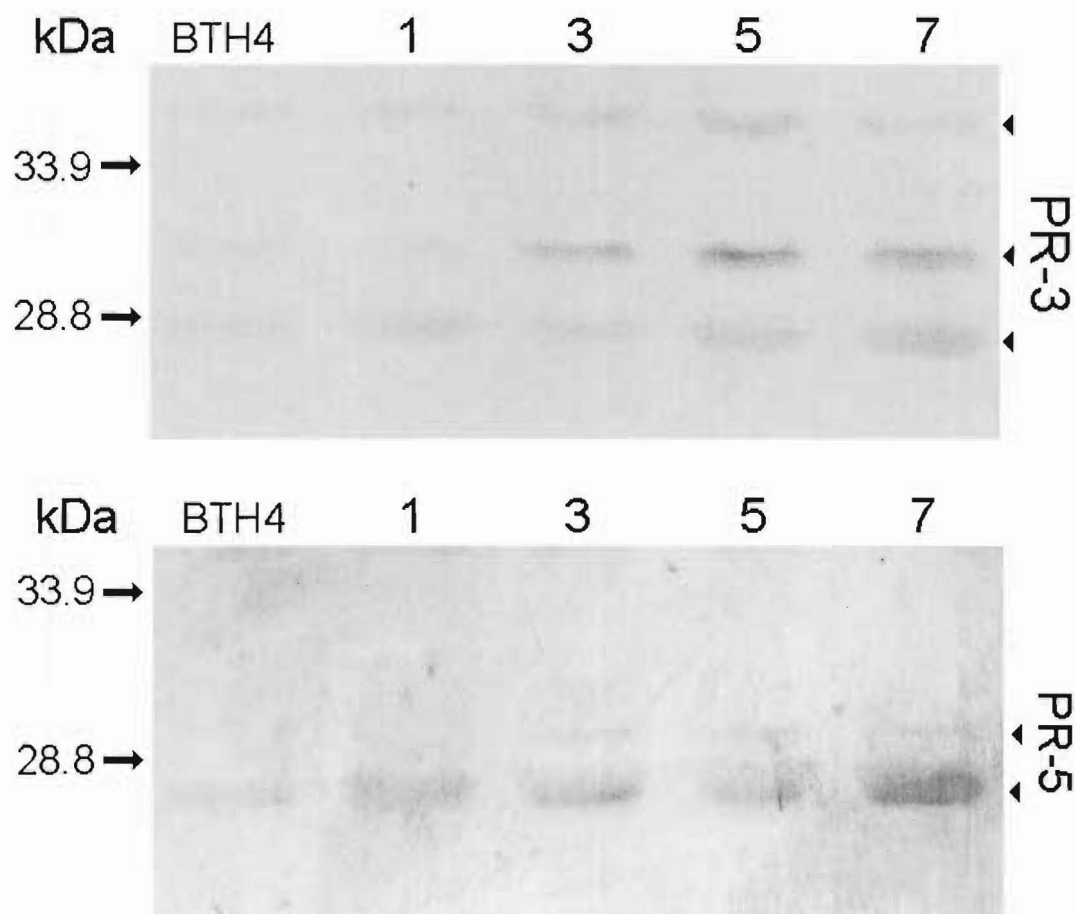


Plate 3.32 The expression of extracellular PR-3 and PR-5 proteins in BTH-pretreated leaves upon inoculation with *D. rosae*.

Whole *in vitro* rose shoots (var. Irish Gee) were first treated with 50 μ M BTH and 4 days later challenge-inoculated with *D. rosae*. Intercellular fluids were extracted at day 0 (lane BTH4), 1, 3, 5, and 7 following inoculation as indicated. After SDS-PAGE, the proteins were subsequently transferred to nitrocellulose membranes. The blots were probed with antisera raised against tobacco PR-Q protein (PR-3 family) and PR-S protein (PR-5 family). Each lane contained 10 μ g of proteins. The molecular weights of prestained marker proteins are indicated on the left in kDa.

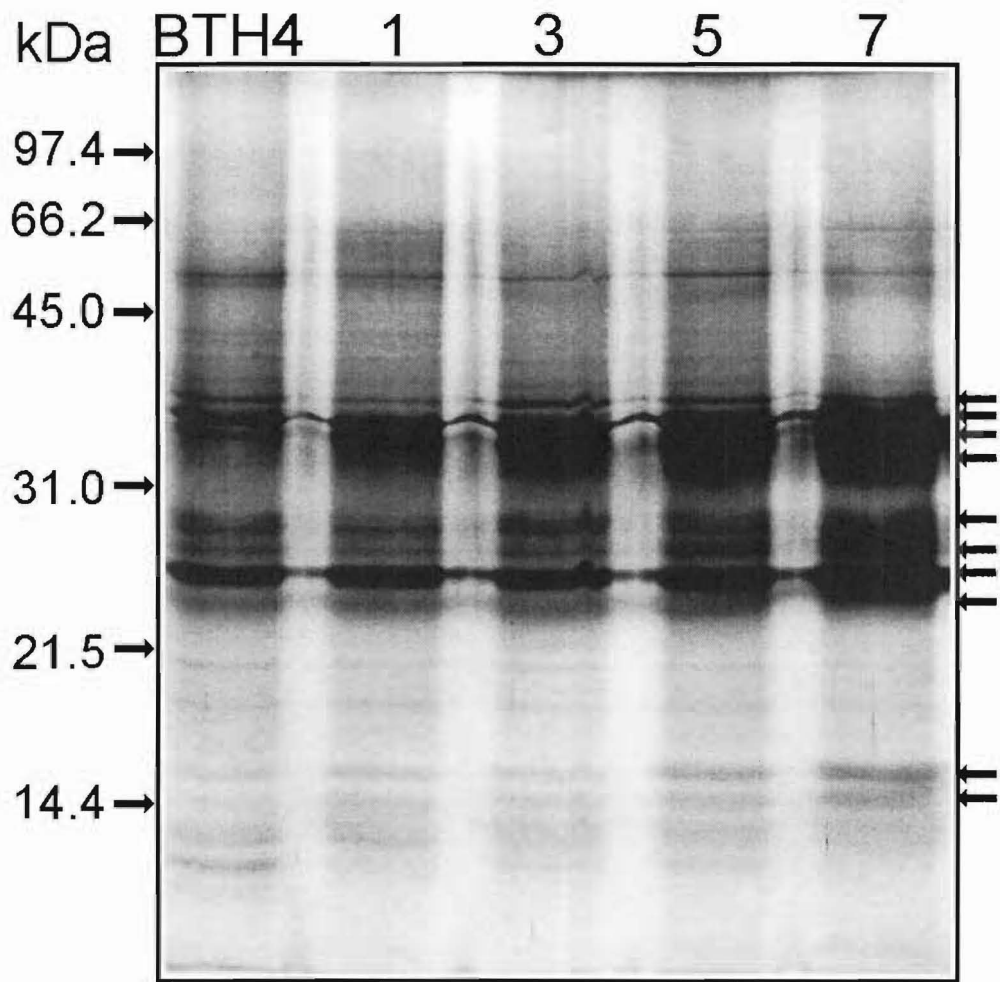


Plate 3.33 SDS-PAGE showing the accumulation of extracellular proteins from BTH-pretreated leaves followed by inoculation with *D. rosae*.

Whole *in vitro* rose shoots (var. Iris Gee) were first treated with 50 μ M BTH and 4 days later challenge-inoculated with *D. rosae*. The intercellular fluids were extracted at day 0 (lane BTH4), 1, 3, 5, and 7 following inoculation as indicated. Each lane was loaded with 5 μ g proteins. After SDS-PAGE, the gel was stained with AgNO_3 . Arrows indicate the proteins that became more abundant after inoculation with *D. rosae*. The molecular weights of marker proteins are indicated on the left in kDa.

3.5 Enzyme activities in the intercellular spaces of rose leaves after BTH treatment

The induction of β -1,3-glucanases and chitinases, the most extensively studied PR proteins, is tightly associated with plant disease resistance (Linthorst 1991, Stintzi et al. 1993, Ryals et al. 1996, Van Loon 1997). In addition, increased peroxidase activity has also been shown to correlate with disease resistance in several crops (Linthorst 1991, Stintzi et al. 1993, Van Loon 1997). To assess whether these enzymes were induced by BTH and how they might be related to the BTH-induced resistance in rose shoots, their activities in intercellular fluids of rose leaves were assayed.

3.5.1 β -1,3-glucanase

Five days after the treatments with different BTH concentrations or water as control, the leaves of rose shoots grown *in vitro* were harvested for extraction of the ICFs. A low level of β -1,3-glucanase activity was found in water-treated leaves. The enzyme activity increased with increasing BTH concentrations (Figure 3.4). The highest BTH concentration (i.e. 5 mM) led to a more than 5-fold increase in the specific β -1,3-glucanase activity over that of the water-treated control.

Following the treatment of rose shoots with 50 μ M BTH, β -1,3-glucanase activity in the ICFs of leaves was determined over a 7-day period (Figure 3.5). The level of the enzyme activity changed little over 7 days in control leaves treated with water. Three days after the BTH treatment, β -1,3-glucanase activity markedly increased. At day 7, in comparison with control, the enzyme activity increased up to 3-fold in the BTH-treated leaves.

Two acidic β -1,3-glucanase isoforms, designated as G1 and G2, were detected when ICFs of BTH-treated leaves were analysed on 15% anodic native PAGE gels (Plate 3.34). The staining intensities of the bands increased with increasing concentrations of BTH. Whereas the activity of β -1,3-glucanases in the water-treated leaves was only barely detectable.

The isoforms G1 and G2 accumulated gradually with time following the treatment with 50 μ M BTH (Plate 3.35). They were faintly detected in the water-treated leaves. It is also interesting to find that the isoform G2, but not G1, was present in the ICF from the yellowing senescent leaves of the *in vitro* rose shoots after a couple months keeping in the shoot multiplication medium (Plate 3.36). The relative levels of G2 in the ICFs were apparently in the following order: senescent leaves > leaves treated with 5 mM BTH for 5 days > water-treated leaves. It has become clear that BTH can stimulate an increase in the specific activity of the isoform G1, which was not detectable in the water-treated or senescent leaves (Plate 3.36).

There was no basic β -1,3-glucanase isoform in the ICF extracts separated on cathodic native PAGE gels (data not shown).

On isoelectric focusing gels (Plate 3.37), the ICF extract from BTH-treated leaves seemed to have three acidic isoforms of β -1,3-glucanases with isoelectric points of 5.7, 5.5 and 4.5 (designated as GI1, GI2 and GI3), respectively. GI2 and GI3 were present in the ICFs of both the water- and BTH-treated leaves, while the band GI1 was induced by BTH.

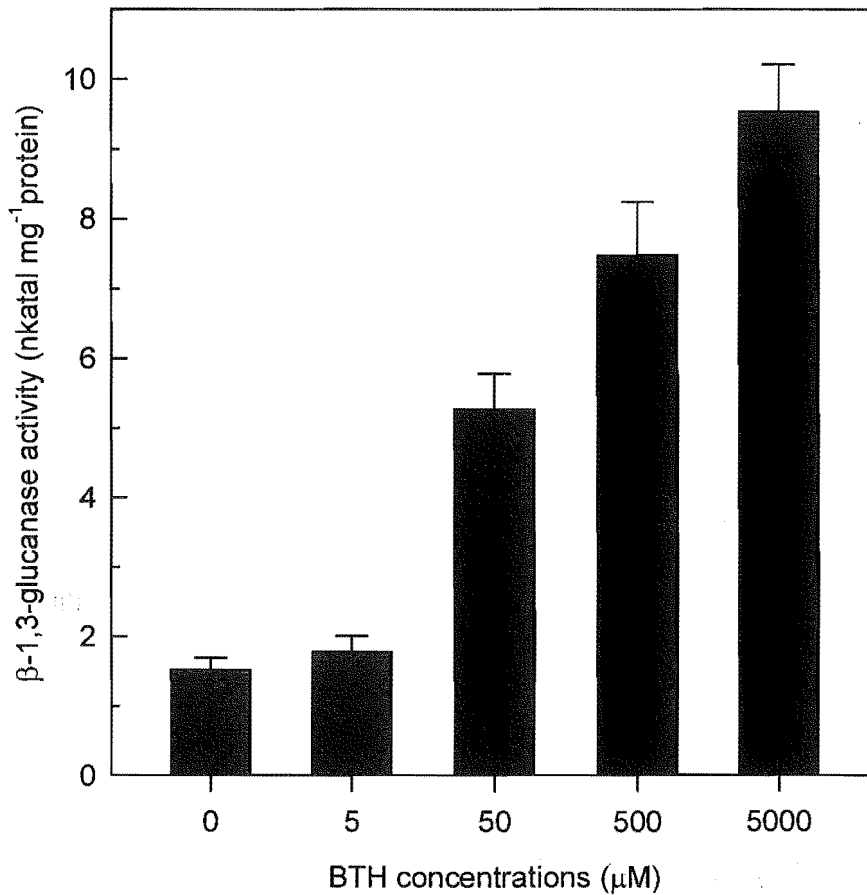


Figure 3.4 Changes of β -1,3-glucanase activity in the intercellular fluids from leaves of *in vitro* rose shoots (var. Iris Gee) after treatment with different concentrations of BTH.

BTH was applied by dipping whole *in vitro* rose shoots (var. Iris Gee) into the chemical at different concentrations or water (0 μM BTH) for a few seconds under aseptic conditions. Five days after the start of the treatments, the leaves were harvested for β -1,3-glucanase activity determination. Each bar represents a mean \pm standard error from three replicates.

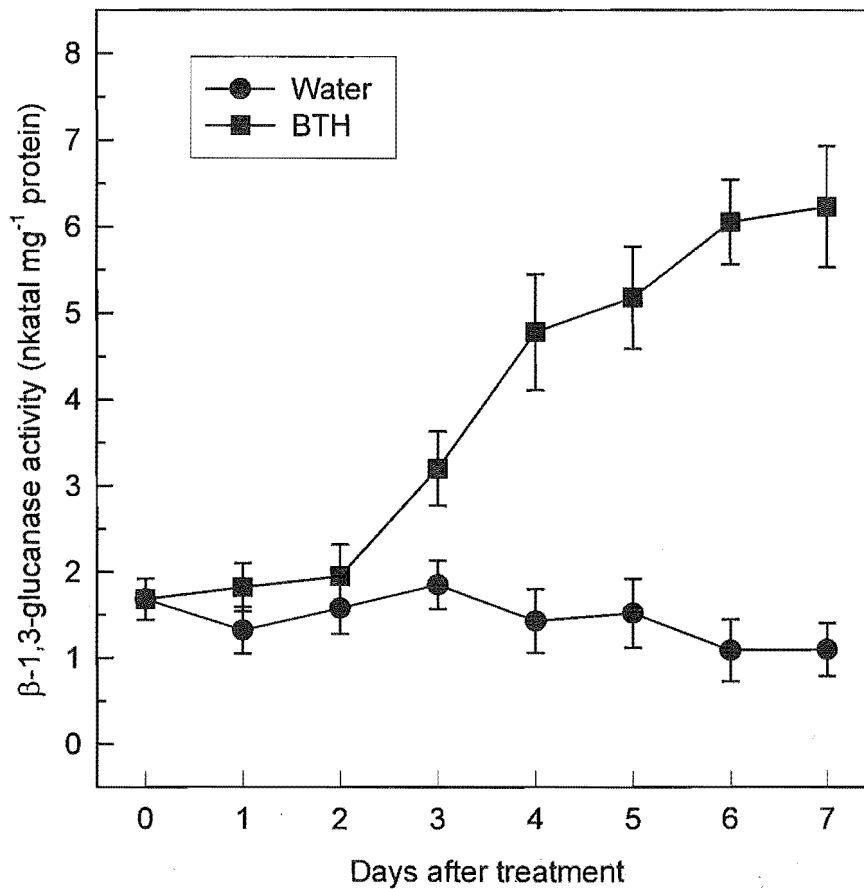


Figure 3.5 Time course of β -1,3-glucanase activity development in the intercellular fluids from leaves of *in vitro* rose shoots (var. Iris Gee) treated with 50 μ M BTH.

In vitro rose shoots (var. Iris Gee) were dipped into either water or BTH at 50 μ M for a few seconds under aseptic conditions before they were returned to the shoot growth medium. At the indicated times after either treatment, the leaves were harvested for β -1,3-glucanase activity determination. Each bar represents a mean \pm standard error from three replicates.

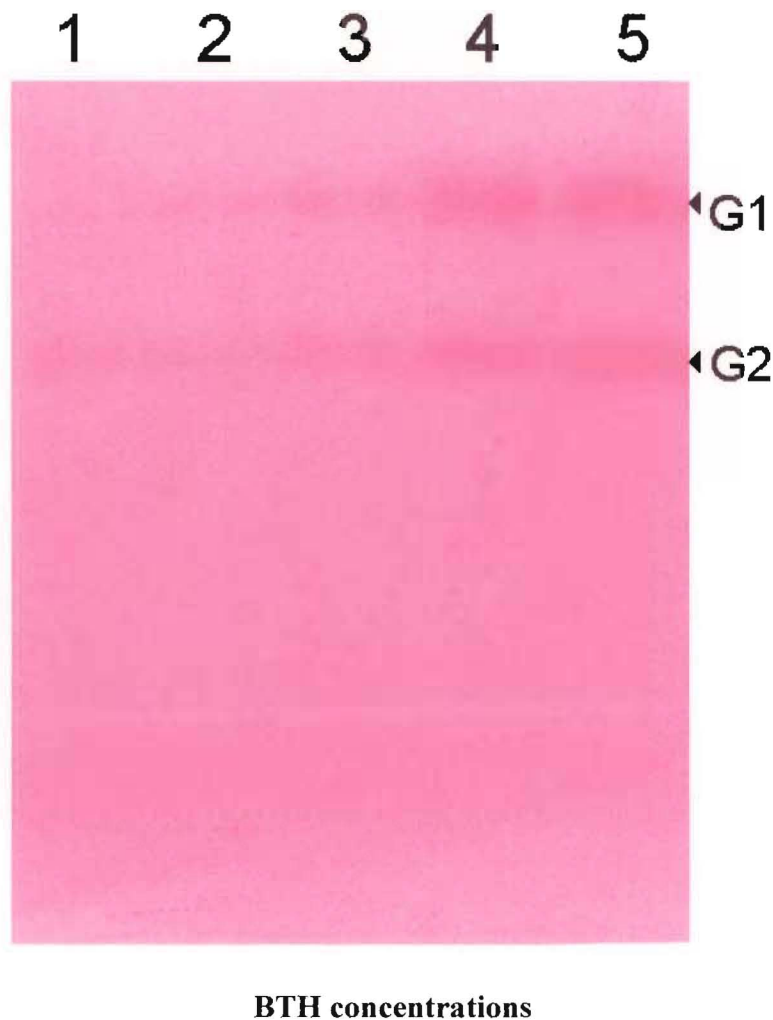


Plate 3.34 Native PAGE analysis of acidic β -1,3-glucanase isoforms in the ICFs from leaves of *in vitro* rose shoots (var. Iris Gee) after 5 days from treatment with different concentrations of BTH.

ICFs were separated on a 15% anodic native PAGE gel. Each lane was loaded with 10 μ g proteins. Lanes from left to right represent ICFs from: (1) water-treated leaves, i.e. 0 μ M BTH; (2) 5 μ M BTH-treated leaves; (3) 50 μ M BTH-treated leaves; (4) 500 μ M BTH-treated leaves; and (5) 5 mM BTH-treated leaves. The Gel was stained for β -1,3-glucanase activity using laminarin as substrate. Arrows indicate bands with activity.

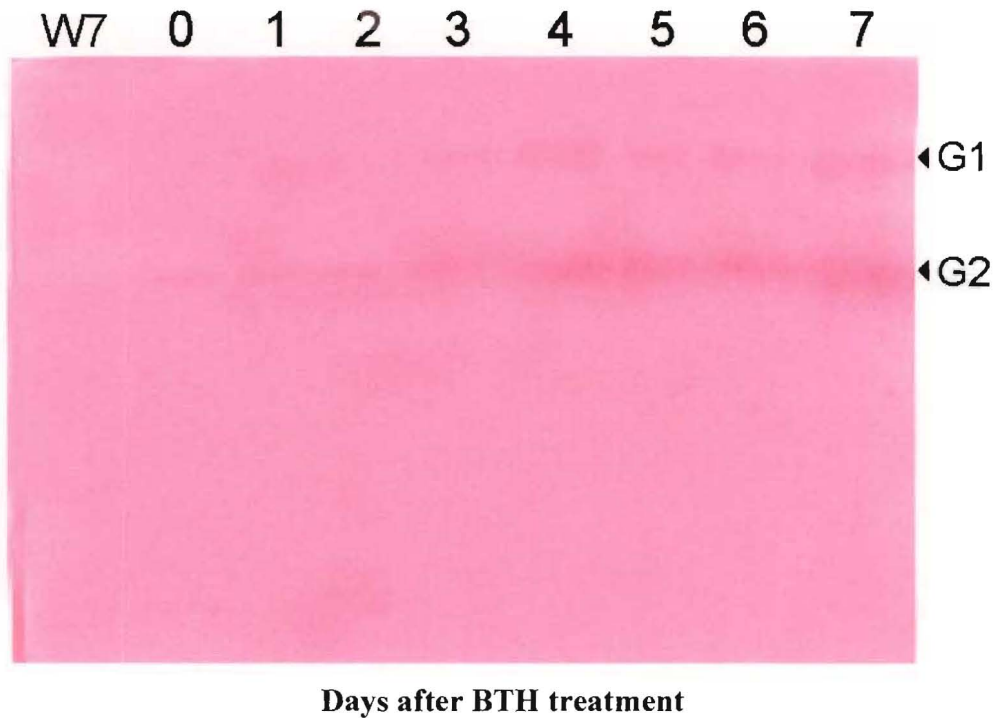


Plate 3.35 Changes of acidic β -1,3-glucanase isoforms over 7 days in the ICFs from leaves of *in vitro* rose shoots (var. Iris Gee) treated with 50 μ M BTH.

ICF extracts (10 μ g protein) were separated on a 15% anodic native PAGE gel. The gel was stained for β -1,3-glucanase activity using laminarin as substrate. W7: water-treated leaves harvested at day 7; Lanes 0-7: leaves were harvested at day 0 to 7 after the treatment with 50 μ M BTH. Arrows indicate bands with activity.

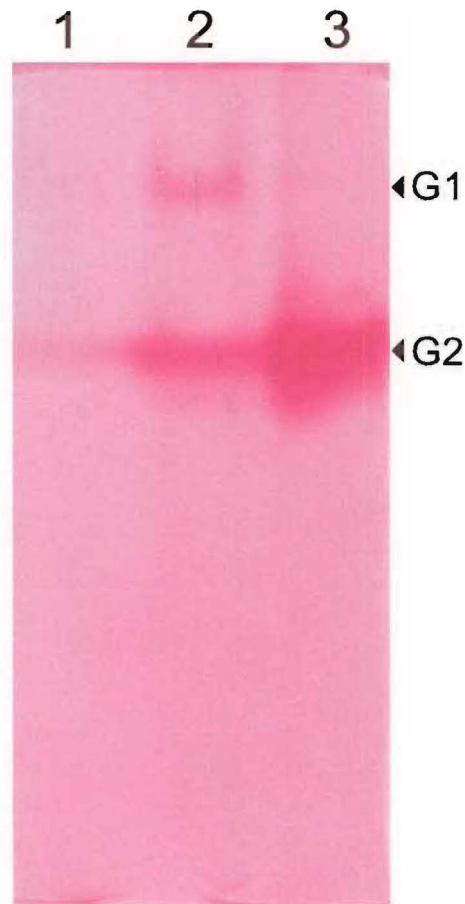


Plate 3.36 A native PAGE gel comparing the acidic β -1,3-glucanase isoforms in the ICFs from leaves of *in vitro* rose shoots (var. Iris Gee) in 3 different treatments.

ICFs were separated on a 15% anodic native PAGE gel. Lane 1: ICF extracted from the leaves after 5 days from dipping in water for a few seconds; Lane 2: extracted from the leaves after 5 days from dipping in 5 mM BTH for a few seconds; Lane 3: extracted from the leaves showing visible signs of senescence (i.e. yellowing) during *in vitro* culture. The gel was stained for β -1,3-glucanase activity using laminarin as substrate. Arrows indicate bands with activity.

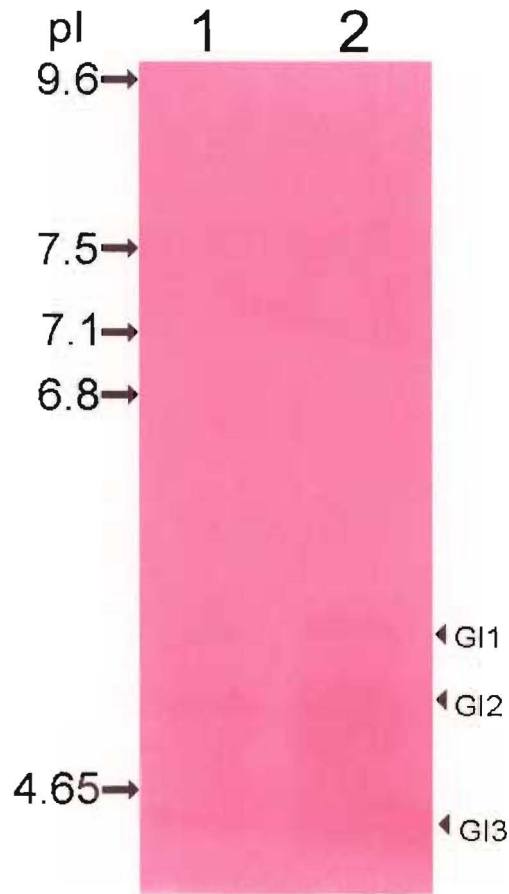


Plate 3.37 IEF gel analysis of β -1,3-glucanase isoform patterns of ICF extracts.

The ICF extracts from the water-treated (lane 1) and the BTH-treated (lane 2) leaves of *in vitro* rose shoots (var. Iris Gee) after 5 days from the start of the treatments were separated on an isoelectric focusing (IEF) gel (pH 3-10). The gel was stained for β -1,3-glucanase activity using laminarin as substrate. The positions of the isoforms (designated as GI1, GI2 and GI3, respectively) are indicated on the right. pI values of IEF markers are shown on the left.

3.5.2 Chitinase

Induction of chitinase activity in ICFs of rose leaves in response to dipping the *in vitro* shoots into different concentrations of BTH showed a dose-dependent pattern (Figure 3.6). Low concentration of BTH (5 μ M) had no effect on the level of chitinase activity in the intercellular spaces of leaves compared with those of the water-treated leaves 5 days after treatments. However, treatment with higher than 50 μ M BTH caused a marked increase in the level of chitinase activity.

Time course of chitinase activity development induced by 50 μ M BTH indicated that after the treatment the enzyme activity in the ICFs increased and started to level off at around day 6 (Figure 3.7). In contrast, there was little change in the enzyme activity in the ICFs of the water-treated control over 7 days.

Native PAGE of ICFs from BTH-treated leaves showed the presence of four acidic chitinase isoforms (designated C1, C2, C3 and C4) running with anodic buffer system (Plate 3.38). The isoforms of C3 and C4 occurred at lower levels in the water-treated control. However, the intensity of these 2 bands increased with increasing BTH concentrations. Additional bands (C1 and C2) were only detected in the leaves treated with the higher concentrations of BTH (50 μ M to 5 mM). Over the 7 days after treatment with 50 μ M BTH, the bands with chitinase activity seemed to increase with time (Plate 3.39). In particular, the isoforms (C1 and C2) appeared at day 4 after the BTH treatment. No basic chitinase isoform was found in ICFs of rose leaves (data not shown).

Analysis by isoelectric focusing revealed that the bands with chitinase activity (designated CI1 and CI2) had pI values of 4.5 and 4.3. They were detected in the ICFs of both water- and BTH-treated leaves (Plate 3.40).

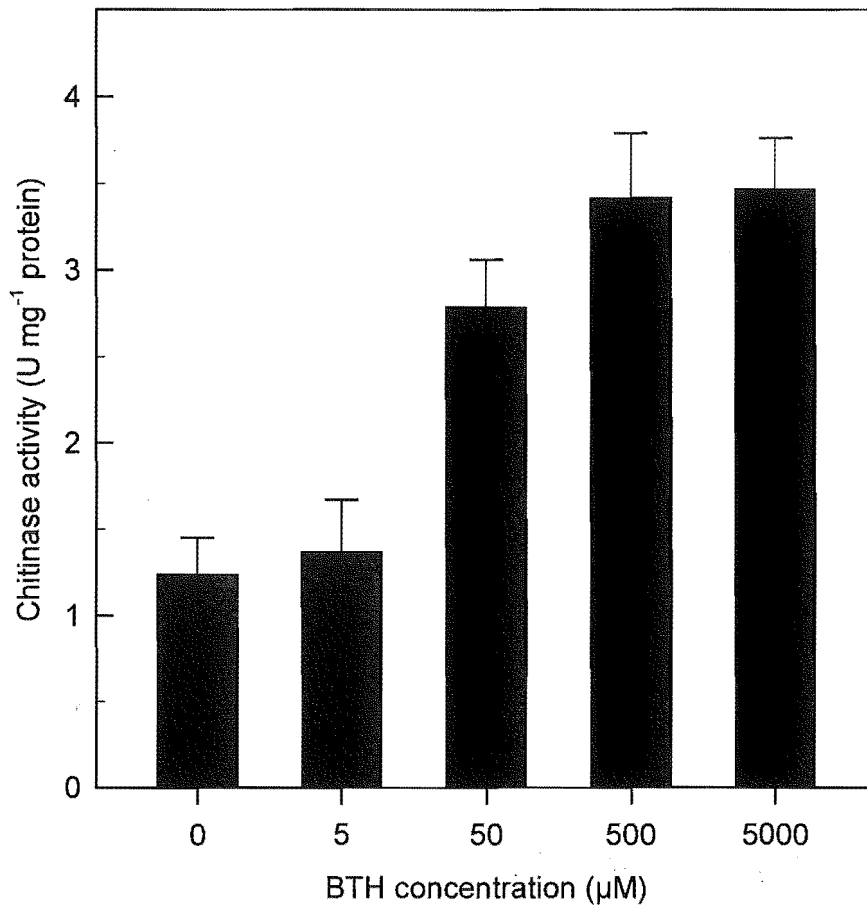


Figure 3.6 Changes of chitinase activity in the intercellular fluids from leaves of *in vitro* rose shoots (var. Iris Gee) after treatment with different concentrations of BTH.

BTH was applied by dipping whole *in vitro* rose shoots (var. Iris Gee) into the chemical at different concentrations or water (0 μM BTH) for a few seconds under aseptic conditions. Five days after the start of the treatments, the leaves were harvested for chitinase activity determination. Each bar represents a mean \pm standard error from three replicates.

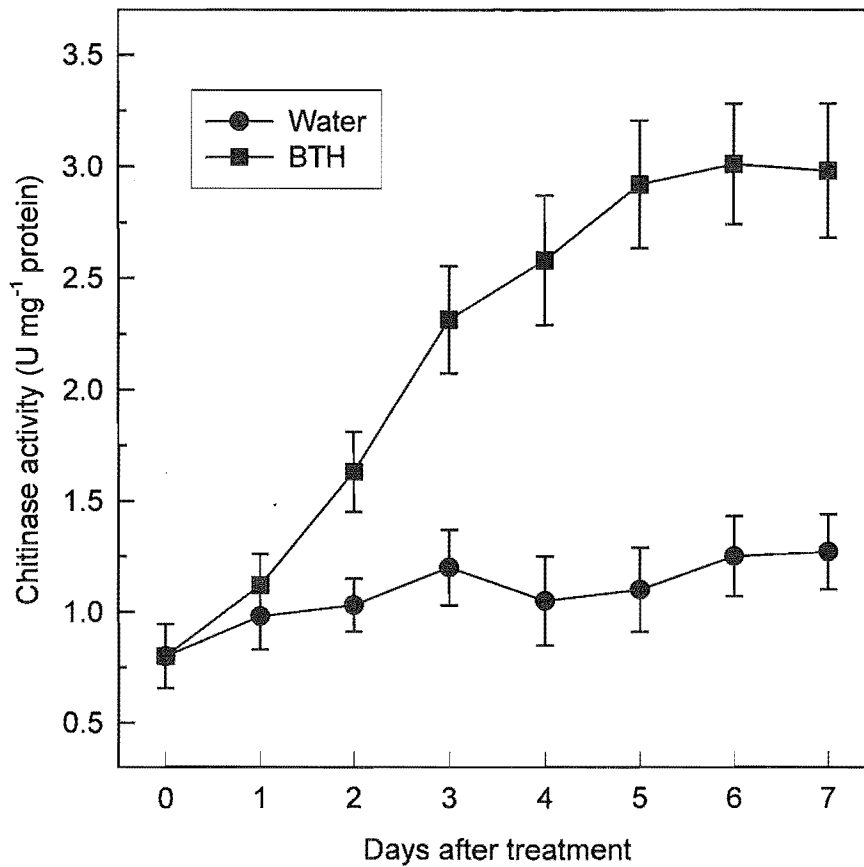
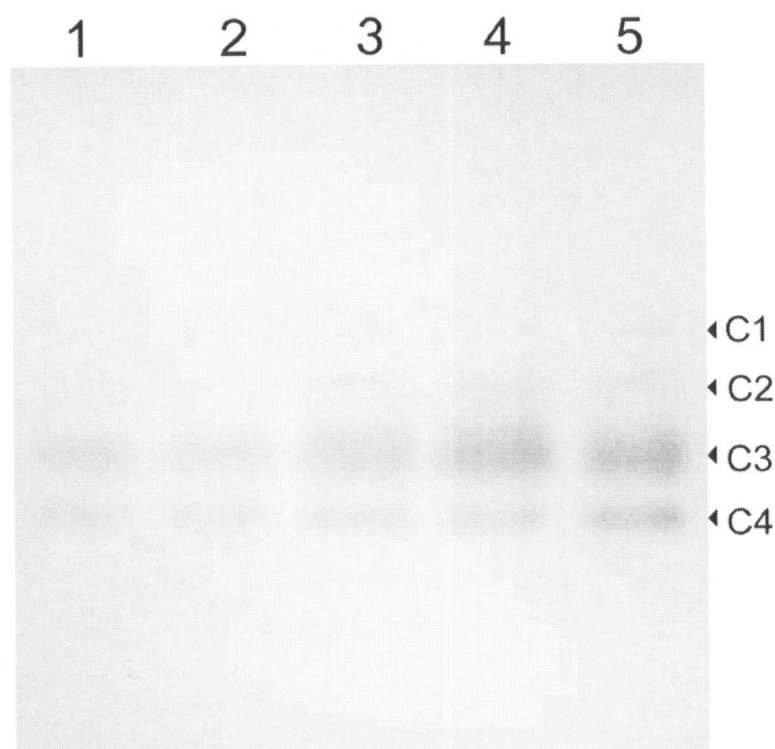


Figure 3.7 Time course of chitinase activity development in the intercellular fluids from leaves of *in vitro* rose shoots (var. Iris Gee) treated 50 μ M BTH.

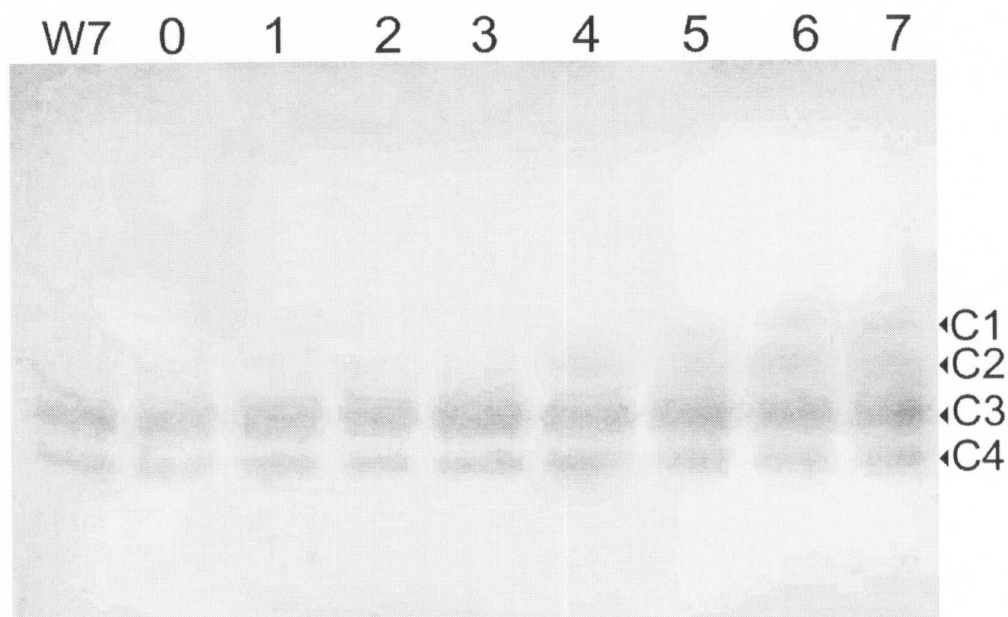
In vitro rose shoots (var. Iris Gee) were dipped into either water or BTH at 50 μ M for a few seconds under aseptic conditions before they were returned to the shoot growth medium. At the indicated times after either treatment, the leaves were harvested for chitinase activity determination. Each bar represents a mean \pm standard error from three replicates.



BTH concentrations

Plate 3.38 Native PAGE analysis of acidic chitinase isoforms in the ICFs from leaves of *in vitro* rose shoots (var. Iris Gee) after 5 days from treatment with different concentrations of BTH.

ICFs were separated on a 10% anodic native PAGE gel. Each lane was loaded with 10 μg proteins. Lanes from left to right represent ICFs from: (1) water-treated leaves, i.e. 0 μM BTH; (2) 5 μM BTH-treated leaves; (3) 50 μM BTH-treated leaves; (4) 500 μM BTH-treated leaves; and (5) 5 mM BTH-treated leaves. The gel was stained for chitinase activity using glycol chitin as substrate. Arrows indicate bands with activity.



Days after BTH treatment

Plate 3.39 Changes of acidic chitinase isoforms over 7 days in the ICFs from leaves of *in vitro* rose shoots (var. Iris Gee) treated with 50 μ M BTH.

ICF extracts (10 μ g protein) were separated on a 10% anodic native PAGE gel. The gel was stained for chitinase activity using glycol chitin as substrate. W7: water-treated leaves harvested at day 7; Lanes 0-7: leaves were harvested at day 0 to 7 after the treatment with 50 μ M BTH. Arrows indicate bands with activity.

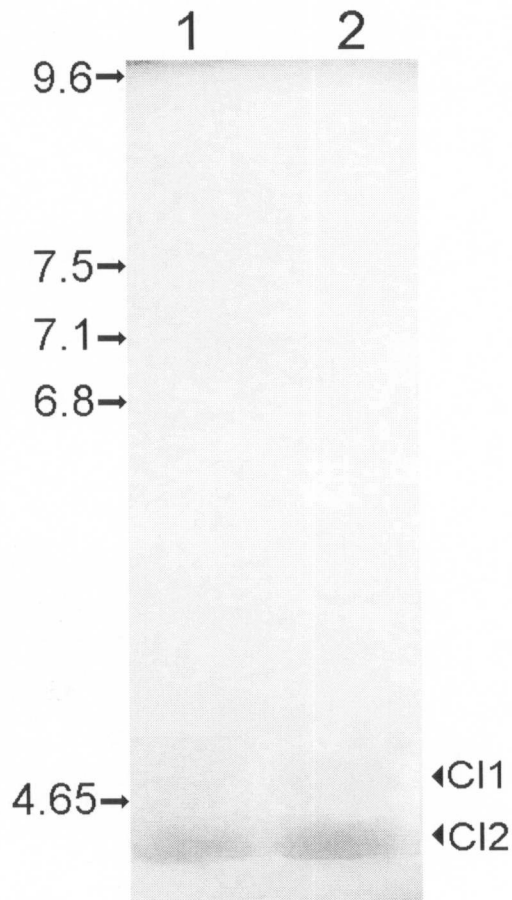


Plate 3.40 IEF gel analysis of chitinase isoform patterns of ICF extracts.

The ICF extracts from the water-treated (lane 1) and the BTH-treated (lane 2) leaves of *in vitro* rose shoots (var. Iris Gee) after 5 days from the start of the treatments were separated on an isoelectric focusing (IEF) gel (pH 3-10), followed by the overlay of a 7.5% polyacrylamide gel containing glycol chitin to detect chitinase isoforms. The positions of the isoforms (designated as CI1 and CI2, respectively) are indicated on the right. pI values of IEF markers are shown on the left.

3.5.3 Peroxidase

The BTH treatment resulted in an increase of the peroxidase activity in the intercellular spaces of rose leaves (Figure 3.8). When rose shoots were treated with 5 mM BTH for 5 days, the enzyme activity was 3-fold higher than that in the control.

The increase of peroxidase activity began almost immediately after the treatment with 50 μ M BTH (Figure 3.9). At day 5, the ICF of the BTH-treated leaves had about 2-fold more peroxidase activity than that in the ICF of the water-treated leaves.

Acidic peroxidase isoforms in the intercellular fluids of rose leaves (var. Iris Gee) were detected on 10% anodic native PAGE gels. Staining of enzyme activity revealed the presence of two acidic peroxidase isoforms (designated as Pa1 and Pa2). The staining intensity of these bands was enhanced by increasing BTH concentrations compared with that of those in the water-treated control (Plate 3.41A).

The isoform Pa2 was first detected in leaves at day 3 after treatment with 50 μ M BTH and then the activity seemed to decline (Plate 3.41B). In contrast, Pa1 changed little with time.

Two basic peroxidase isoforms (designated Pb1 and Pb2) were detected in ICF extracts of rose leaves on 10% cathodic native PAGE gels (Plate 3.42A). Pb1 appeared to be more prominent than Pb2. The staining intensity of the peroxidase bands was more pronounced in the extracts of BTH-treated leaves than that of the controls. Over 7 days after treatment with 50 μ M BTH, the peroxidase isoforms were either not detected or only faintly detected in control, but Pb1 was strongly detected from day 4 onwards after the BTH treatment, while Pb2 was induced at 1 day after the BTH treatment (Plate 3.42B).

Analysis by isoelectric focusing (IEF) showed that the intercellular fluids contained both cationic and anionic peroxidase isoforms (Plate 3.43). The isoelectric points of the isoforms were about 8.9 and below 4, respectively.

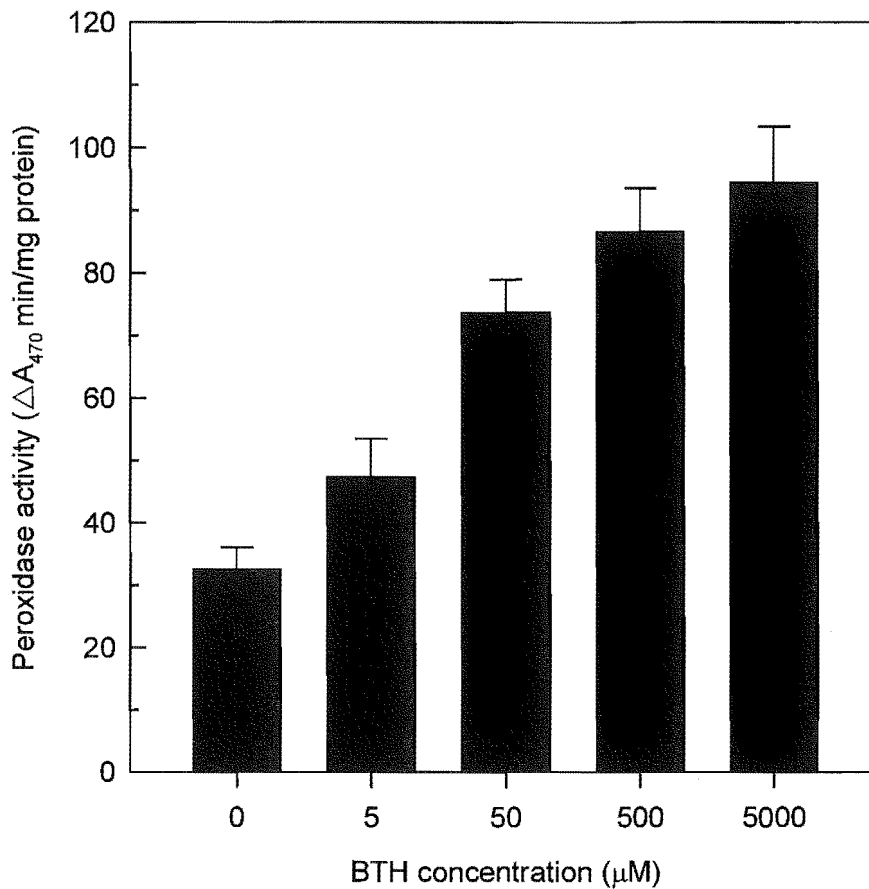


Figure 3.8 Changes of peroxidase activity in the intercellular fluids from leaves of *in vitro* rose shoots (var. Iris Gee) after treatment with different concentrations of BTH.

BTH was applied by dipping whole *in vitro* rose shoots (var. Iris Gee) into the chemical at different concentrations or water (0 μM BTH) for a few seconds under aseptic conditions. Five days after the start of the treatments, the leaves were harvested for peroxidase activity determination. Each bar represents a mean \pm standard error from three replicates.

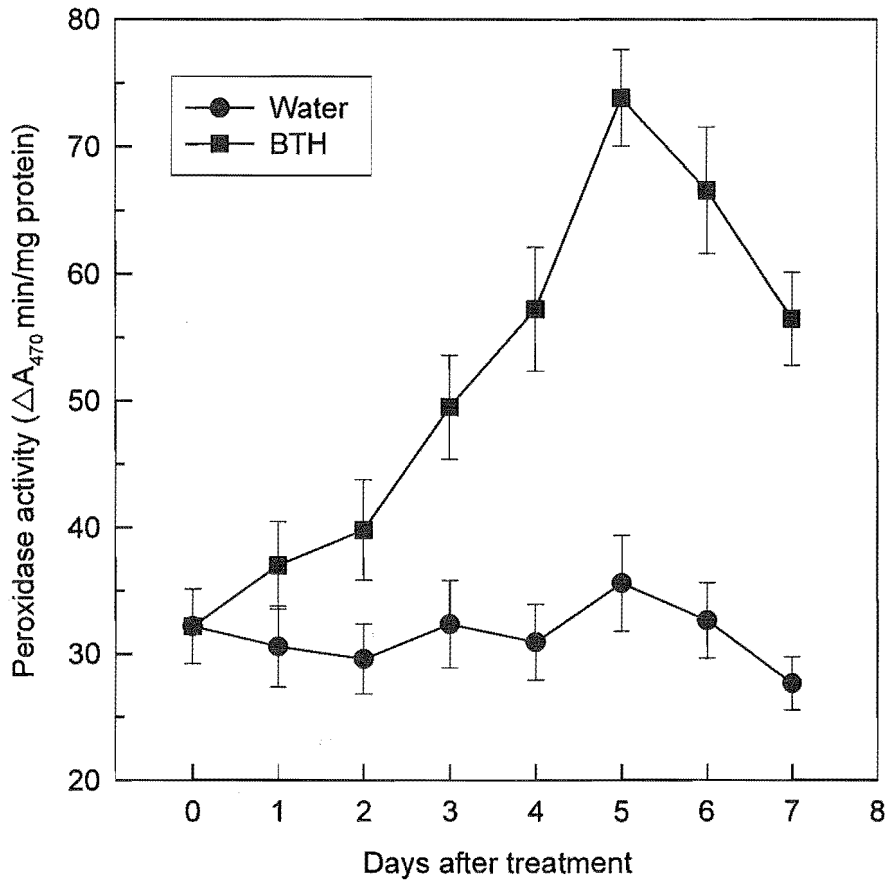


Figure 3.9 Time course of peroxidase activity development in the intercellular fluids from leaves of *in vitro* rose shoots (var. Iris Gee) treated with 50 μ M BTH.

In vitro rose shoots (var. Iris Gee) were dipped into either water or 50 μ M BTH for a few seconds under aseptic conditions before they were returned to the shoot growth medium. At the indicated times after either treatment, the leaves were harvested for peroxidase activity determination. Each bar represents a mean \pm standard error from three replicates.

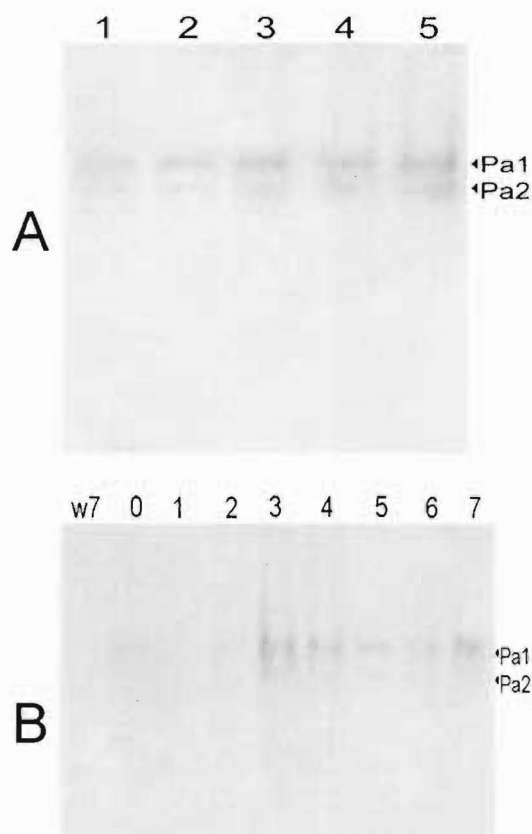


Plate 3.41 Native PAGE analysis of acidic peroxidase isoforms in the ICFs from water- or BTH-treated leaves of *in vitro* rose shoots (var. Iris Gee).

(A) Rose shoots grown *in vitro* were treated with various concentrations of BTH and ICFs of the leaves were obtained 5 days after the treatments. Lanes from left to right represent ICFs from: (1) water-treated leaves; (2) 5 μ M BTH-treated leaves; (3) 50 μ M BTH-treated leaves; (4) 500 μ M BTH-treated leaves; and (5) 5 mM BTH-treated leaves. (B) The ICFs of leaves were obtained at the indicated times after treatment with 50 μ M BTH over 7 days. W7: the ICF of leaves was obtained at day 7 after water treatment. The gels were stained for peroxidase activity using o-dianisidine as substrate. Arrows indicate bands with activity.

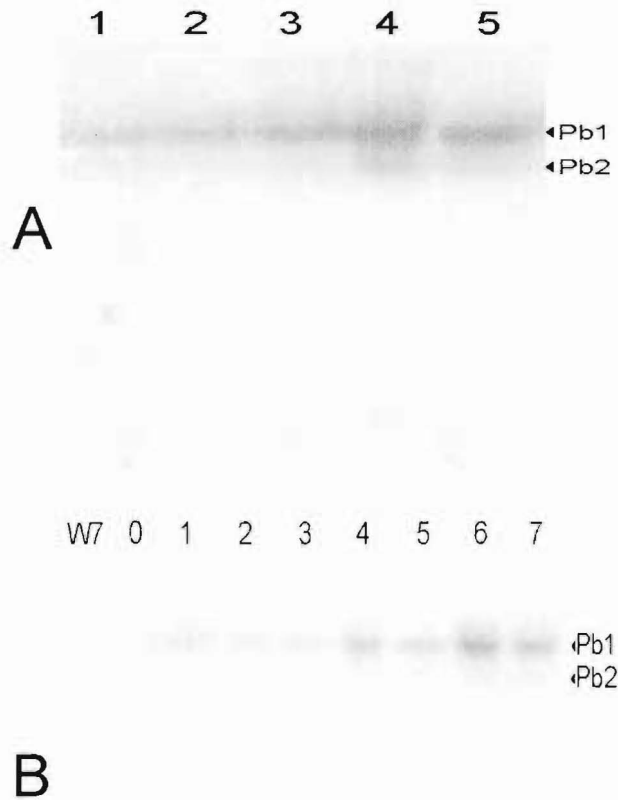


Plate 3.42 Native PAGE analysis of basic peroxidase isoforms in the ICFs from water- or BTH-treated leaves of *in vitro* rose shoots (var. Iris Gee).

(A) Rose shoots grown *in vitro* were treated with various concentrations of BTH and ICFs of the leaves were obtained 5 days after the treatments. Lanes from left to right represent ICFs from: (1) water-treated leaves; (2) 5 μ M BTH-treated leaves; (3) 50 μ M BTH-treated leaves; (4) 500 μ M BTH-treated leaves; and (5) 5 mM BTH-treated leaves. (B) The ICFs of leaves were obtained at the indicated times after treatment with 50 μ M BTH over 7 days. W7: the ICF of leaves was obtained at day 7 after water treatment. The gels were stained for peroxidase activity using o-dianisidine as substrate. Arrows indicate bands with activity.

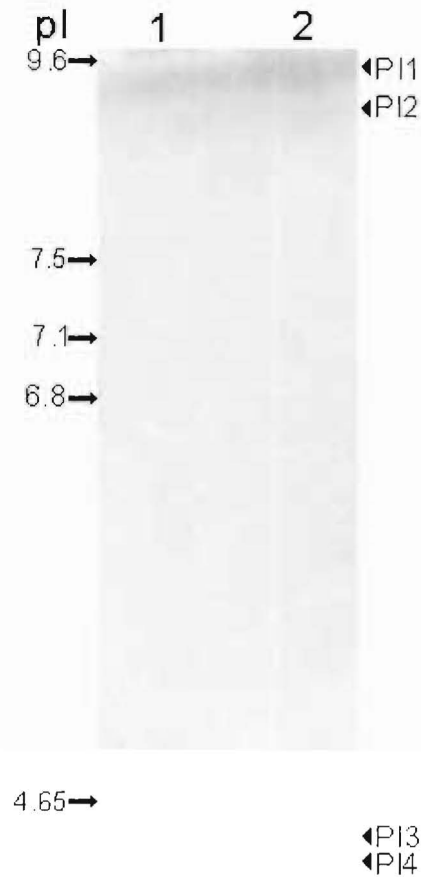


Plate 3.43 IEF gel analysis of peroxidase isoform patterns of ICF extracts.

The ICF extracts from the water-treated (lane 1) and the BTH-treated (lane 2) leaves of *in vitro* rose shoots (var. Iris Gee) after 5 days from the start of the treatments were separated on an isoelectric focusing (IEF) gel (pH 3-10). The gel was stained for peroxidase activity using o-dianisidine as substrate. The positions of the isoforms (designated as PI1, PI2, PI3 and PI4, respectively) are indicated on the right. pI values of IEF markers are shown on the left.

3.6 Enzyme activities in the intercellular spaces of rose leaves with or without pretreatment with BTH followed by challenge inoculation with *D. rosae*

Chitinase, β -1,3-glucanase and/or peroxidase are thought to be involved in plant defence reaction in many plant species in response to pathogen infection (Bol et al. 1990, Linthorst 1991, Stintzi et al. 1993). In rose, this remains to be demonstrated.

After the inoculation of rose leaves with a conidial suspension of *D. rosae*, β -1,3-glucanase, chitinase, and peroxidase activities in ICF extracts from water-pretreated and 50 μ M BTH-pretreated leaves were investigated over a period of 7 days.

3.6.1 β -1,3-glucanase

The level of extracellular β -1,3-glucanase in rose leaves pretreated with water and then infected with *D. rosae* increased throughout the experimental period (Figure 3.10). In the BTH-pretreated leaves followed by *D. rosae* inoculation, the activity of β -1,3-glucanase was stimulated further, reaching a maximum level at day 3 after inoculation with the pathogen (Figure 3.10).

There was no difference in the acidic isoform patterns of β -1,3-glucanase activity in the ICFs extracted from the blackspot-infected rose leaves between the pretreatment with and without 50 μ M BTH on non-denaturing PAGE gel (Plate 3.44). The activity of β -1,3-glucanase in BTH-pretreated leaves was noticeably higher than that in the leaves pretreated with water in response to infection with *D. rosae*. In particular, the isoform (G 1) accumulated to a relatively high level in BTH + *D. rosae* treatment at day 3 after inoculation.

3.6.2 Chitinase

The activity of chitinase in the intercellular spaces of rose leaves pretreated with water increased strongly after the infection with *D. rosae* (Figure 3.11). Chitinase activity increased starting at day 1 when the spores start to germinate on the leaves. It continued to increase to a higher level at day 7 when the disease symptoms were fully developed. Whereas in the BTH + *D. rosae* treatment, the enzyme activity markedly increased by day 3 and continued to increase to nearly 2 times compared with that in water + *D. rosae* treatment at day 5 (Figure 3.11).

Acidic chitinase isoforms in the ICF extracts of water-treated and/or BTH-treated leaves of *in vitro* rose shoots were detected at various times after inoculation with *D. rosae*. Four acidic isoforms (designated C1, C2, C3 and C4) were found in blackspot-infected and BTH-treated leaves (Plate 3.45). C1 and C2 were undetectable in the water-treated, healthy control. The pretreatment with BTH seemed to enhance the staining intensities of the chitinase bands (C3 and C4) in response to the pathogen infection.

3.6.3 Peroxidase

Rose shoots infected with blackspot did not lead to the marked increase of peroxidase activity in the intercellular fluids of leaves pretreated with water or 50 μ M BTH over the period of 7 days after inoculation (data not shown).

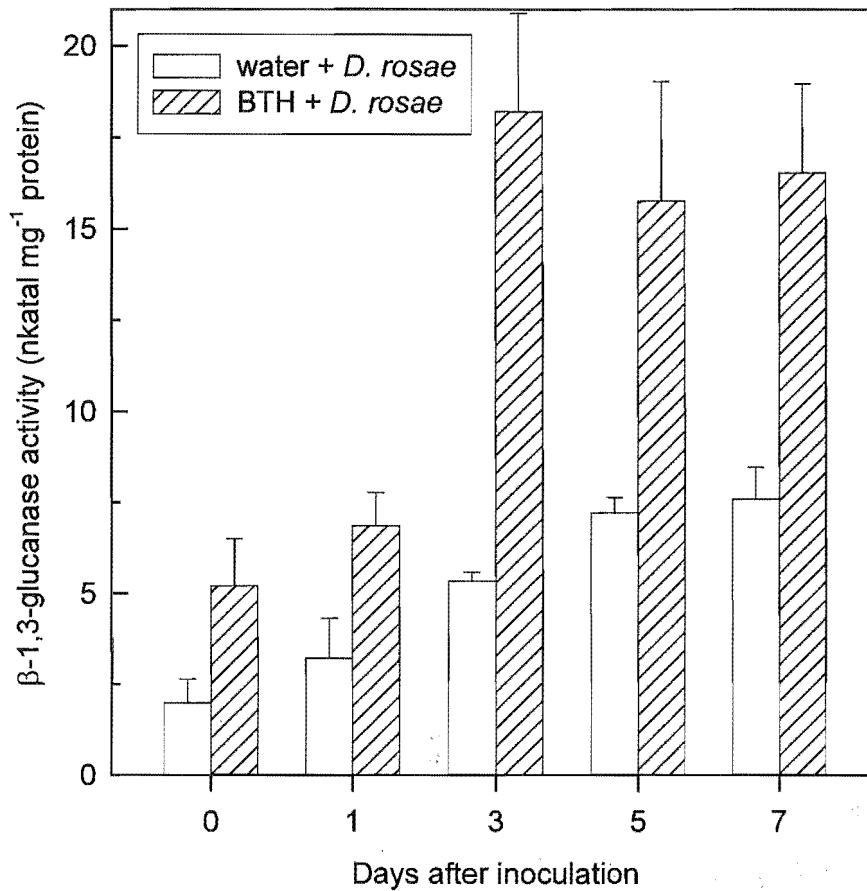


Figure 3.10 Changes of β -1,3-glucanase activity in the intercellular fluids of BTH- or water-pretreated leaves of *in vitro* rose shoots (var. Iris Gee) followed by inoculation with *D. rosae*.

Rose shoots grown *in vitro* were first treated with either water or 50 μM BTH. Four days later they were challenge-inoculated with *D. rosae* by dipping them into a conidial suspension. At the indicated times after inoculation, the leaves were harvested for β -1,3-glucanase activity determination. Each bar represents mean \pm standard error from one experiment, each in triplicate.

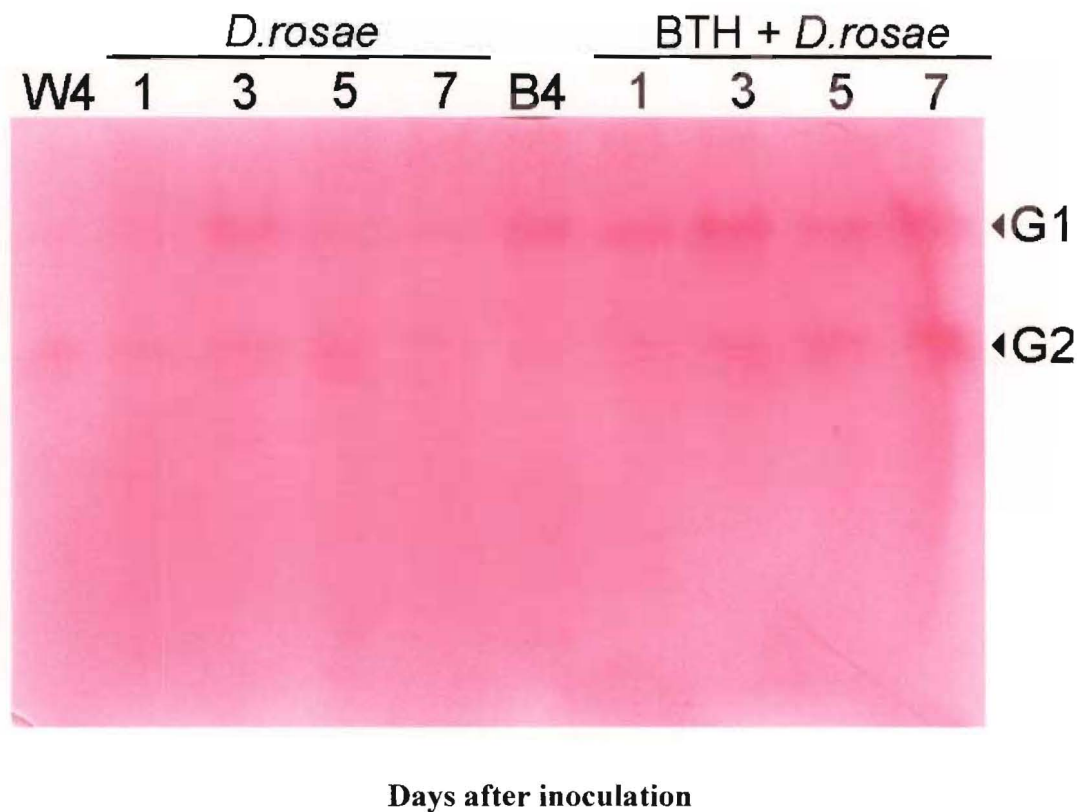


Plate 3.44 Native PAGE analysis of acidic β -1,3-glucanase isoforms in the ICFs from BTH- or water-pretreated leaves of *in vitro* rose shoots (var. Iris Gee) after inoculation with *D. rosae*.

ICF extracts from the leaves of *in vitro* rose shoots 4 days after treatment with water (W4) or 50 μ M BTH (B4), and subsequently from the leaves at day 1, 3, 5, and 7 after inoculation with *D. rosae* as indicated were loaded on a 15% anodic native PAGE gel. The gel was stained for β -1,3-glucanase activity using laminarin as substrate. The positions of the isoforms are indicated on the right.

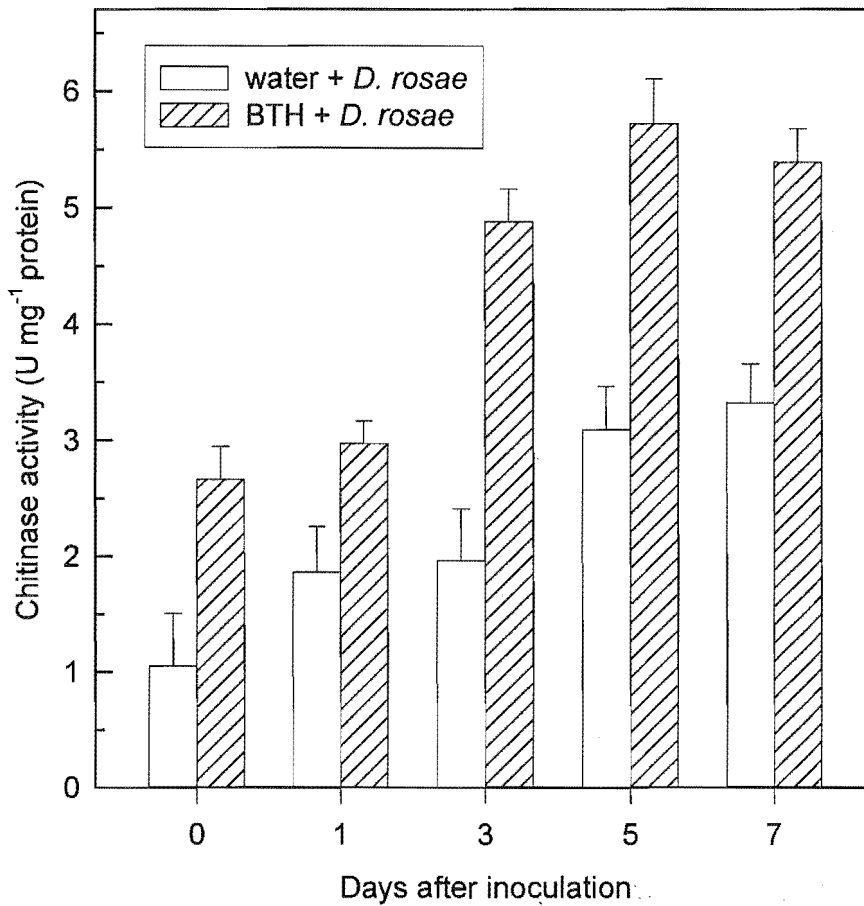


Figure 3.11 Changes of chitinase activity in the intercellular fluids of BTH- or water-pretreated leaves of *in vitro* rose shoots (var. Iris Gee) followed by inoculation with *D. rosae*.

Rose shoots grown *in vitro* were first treated with either water or 50 μ M BTH. Four days later they were challenge-inoculated with *D. rosae* by dipping them into a conidial suspension. At the indicated times after inoculation, the leaves were harvested for chitinase activity determination. Each bar represents mean \pm standard error from one experiment, each in triplicate.

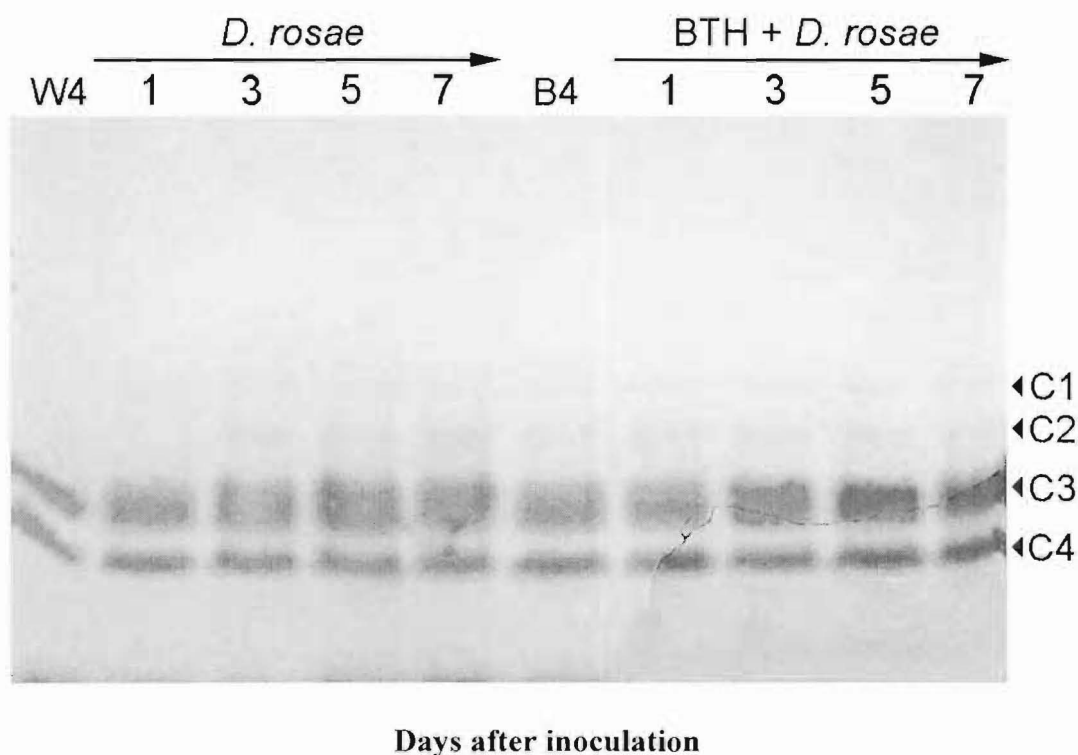


Plate 3.45 Native PAGE analysis of acidic chitinase isoforms in the ICFs from BTH- or water-pretreated leaves of *in vitro* rose shoots (var. Iris Gee) after inoculation with *D. rosae*.

ICF extracts from the leaves of *in vitro* rose shoots 4 days after treatment with water (W4) or 50 μ M BTH (B4), and subsequently from the leaves at day 1, 3, 5, and 7 after inoculation with *D. rosae* as indicated were loaded on a 10% anodic native PAGE gel. The gel was stained for chitinase activity using glycol chitin as substrate. The positions of the isoforms are indicated on the right.

3.7 The expression of extracellular PR proteins and their regulation in response to a range of treatments

In order to ascertain whether BTH specifically induced the PR proteins, the possibility that the PR proteins in the four major classes were also induced and regulated by abiotic inducing agents in rose was investigated. The ICFs extracted from the leaves of *in vitro* rose shoots 5 days after the treatment with following chemicals: 1.5 mM salicylic acid (SA), 10 mM ethephon, 0.3 mM HgCl₂, 50 µM or 5 mM BTH, were subjected to western blot analysis. For comparison, the ICF extracted from the leaves 7 days after blackspot infection was similarly analysed. In addition, the possibility that the PR proteins in different classes are developmentally regulated in rose was also investigated, using the ICF extract of senescent leaves that were visibly yellow during *in vitro* culture. The controls included the ICF extracts from the leaves of those shoots treated with water and the third and fourth compound leaves of one-month-old healthy rose pot plants.

3.7.1 Effect of chemical treatment on shoot appearance

Five days after the treatments, the application of 1.5 mM SA and 50 µM BTH did not affect the appearance of rose shoots. However, the leaves of shoots treated with 10 mM ethephon and 0.3 mM HgCl₂ turned yellowish and developed larger necrotic lesions, respectively (data not shown). The retarded growth of shoots was also observed in the treatment of 5 mM BTH.

3.7.2 Immunological detection of extracellular PR proteins

No PR-1 bands were detected in the ICFs from leaves of pot plants and the leaves treated with HgCl₂, while low expression of 27 and 38 kDa bands was found in leaves treated with 1.5 mM SA or water (Plate 3.46). It is interesting to note that BTH,

ethephon, blackspot-infected leaves and senescent leaves all induced the 15 kDa PR-1 band. However, 1.5 mM SA did not induce the 15 kDa PR-1 protein at a detectable level. It seems that the 38 kDa protein was more strongly induced in senescent leaves.

PR-2 proteins were undetectable in the pot plant control (Plate 3.46). The 26 kDa and 36 kDa isoforms of PR-2 were present at higher levels in the treatments of salicylic acid, ethephon, BTH, blackspot infection and during leaf senescence than in the leaves of healthy *in vitro* shoots, i.e. water-treated leaves. Furthermore, an additional band, i.e. 37 kDa PR-2 protein, was only detected in BTH-treated and blackspot-infected leaves.

At least three PR-3 proteins were found in the extracts from all treatments except from rose pot plants (Plate 3.47). However, some differences exist among the treatments. The water-treated and SA-treated leaves each had relatively low levels of the 25 and 33 kDa PR-3 protein expression. In contrast, these PR-3 proteins were strongly induced by the treatments with ethephon, HgCl_2 , BTH, and blackspot infection as well as in senescent leaves, particularly, the additional band of 37.5 kDa. However, the intensity of the 25 and 33 kDa bands was stronger in the ethephon-treated and senescent leaves.

Two proteins with molecular weights of 25.5 and 27.5 kDa in the ICFs from the treated leaves cross-reacted with antiserum raised against tobacco PR-S except from leaves of pot plants and water-treated leaves (Plate 3.47). The intensity of the PR-5 proteins was higher in the treatments with ethephon, BTH, blackspot infection and in senescent leaves.

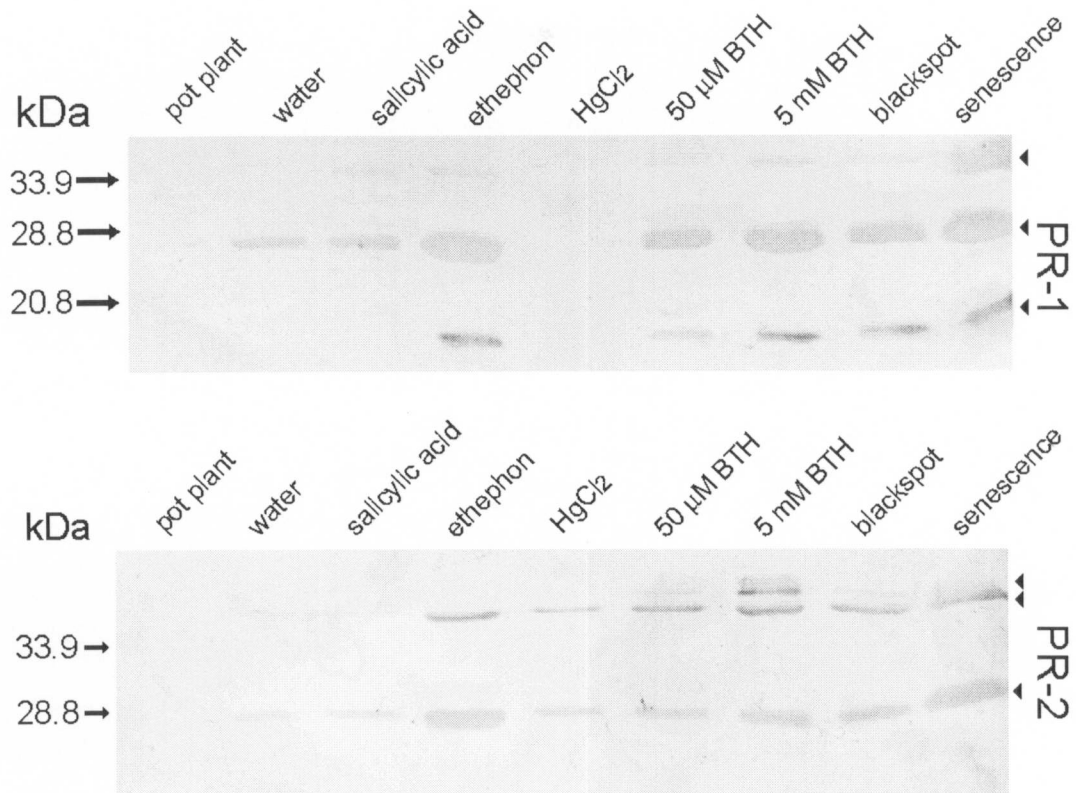


Plate 3.46 Western-blot analysis of the accumulation of extracellular PR-1 and PR-2 proteins in rose leaves following different treatments.

Whole *in vitro* rose shoots (var. Iris Gee) were dipped into the following solutions, one for each treatment: 1.5 mM salicylic acid, 10 mM ethephon, 0.3 mM HgCl₂, 50 μ M BTH, 5 mM BTH and water for a few seconds before returning them to the shoot growth medium. ICFs were extracted from the leaves 5 days after the chemical treatments, blackspot-infected leaves 7 days after inoculation and senescent leaves that were visibly yellow during *in vitro* culture. After SDS-PAGE, the proteins were transferred to nitrocellulose membranes. The blots were probed with antisera raised against tobacco PR-1c protein (PR-1 family) and PR-N protein (PR-2 family). The molecular weights of prestained marker proteins are indicated on the left in kDa.

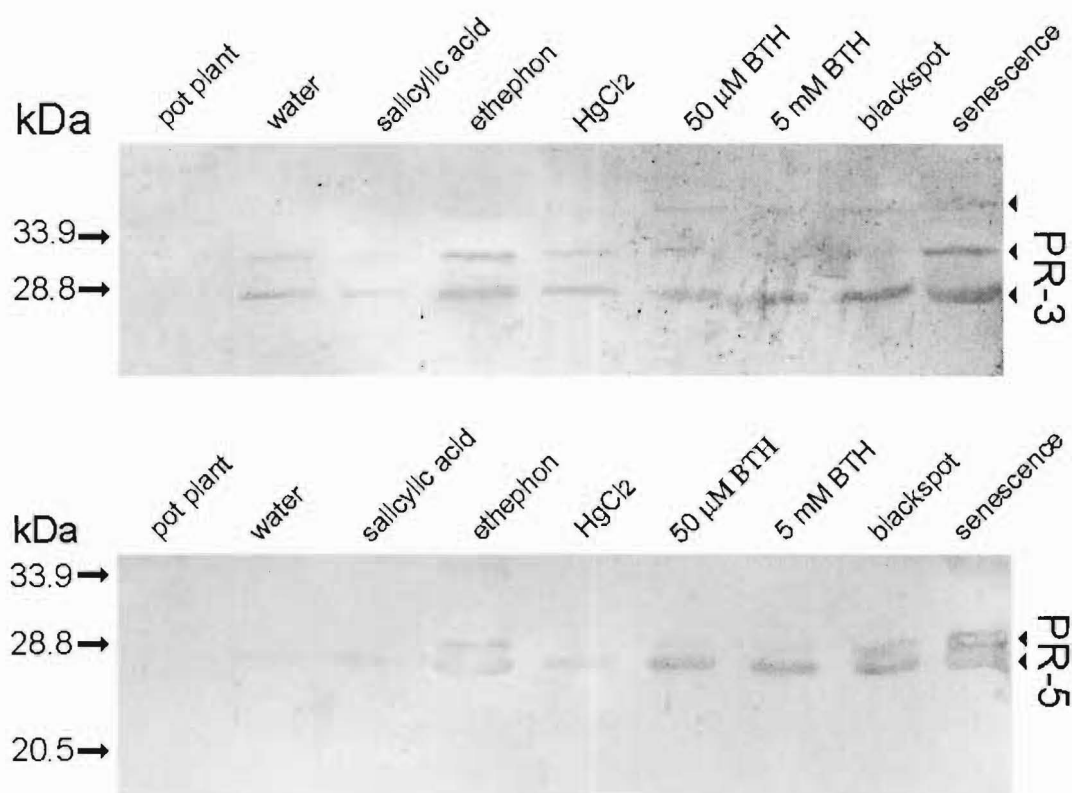


Plate 3.47 Western-blot analysis of the accumulation of extracellular PR-3 and PR-5 proteins in rose leaves following different treatments.

Whole *in vitro* rose shoots (var. Iris Gee) were dipped into the following solutions, one for each treatment: 1.5 mM salicylic acid, 10 mM ethephon, 0.3 mM HgCl₂, 50 μM BTH, 5 mM BTH and water for a few seconds before returning them to the shoot growth medium. ICFs were extracted from the leaves 5 days after the chemical treatments, blackspot-infected leaves 7 days after inoculation, and senescent leaves that were visibly yellow during *in vitro* culture. After SDS-PAGE, the proteins were transferred to nitrocellulose membranes. The Blots were probed with antisera raised against tobacco PR-Q protein (PR-3 family) and PR-S protein (PR-5 family). The molecular weights of prestained marker proteins are indicated on the left in kDa.

3.8 The effect of intercellular fluid from BTH-treated leaves on fungal growth *in vitro*

Intercellular fluid extracted from the leaves of *in vitro* rose shoots after 5 days from treatment with 50 μ M BTH was tested against the growth of *D. rosae* by the method of microtitre plates. A solution containing 30 μ g protein (ICF or boiled ICF) were added to a well containing PDA with the conidial suspension of *D. rosae*. Six days after incubation, it was shown that the addition of protein solution had no effect on the growth of *D. rosae* (data not shown).

Chapter 4

DISCUSSION

4.1 Induction of disease resistance in response to BTH treatment

4.1.1 *The advantage of in vitro rose shoots for studies on response to pathogens*

In the present study, the experiments on BTH treatments and challenge inoculation were all carried out *in vitro*. It is a quick and simple method for studying resistance to *D. rosae* or *A. tumefaciens* in rose. For example, visible symptoms of blackspot develop on rose pot plants at 10-14 day after inoculation under control conditions (Xue and Davidson 1998), whereas full development of blackspot only takes 7 days on leaves of rose shoots *in vitro*.

In all the experiments here, growth of fungus was not visible on the 0.8% agar medium, indicating that the present method of fungal pathogen inoculation did not affect the test results as the inoculated shoots still produced roots. This also demonstrated that the shoots inoculated under *in vitro* conditions can be used, at least as a simple and initial screen, to evaluate resistance to pathogens among rose cultivars.

4.1.2 Response of *in vitro* rose shoots to BTH application and inoculation with two pathogens

It has been reported that rose plants can acquire resistance to *Sphaerotheca pannosa* after INA treatment (Hijwegen et al. 1996). In the present study, it was demonstrated for the first time that after BTH treatment rose shoots could develop an increased resistance against the other two pathogens, i.e., *D. rosae* and *A. tumefaciens* which are pathogens of agricultural importance in rose. *In vitro* antifungal or antibacterial assay demonstrated that BTH has no direct antimicrobial activity against these two pathogens. But, BTH significantly induced resistance, indicating that BTH seems to work by activating rose plant defence mechanism against the pathogens rather than as an antibiotic *in vitro*. This supports the concept that BTH is probably a potential disease-control chemical in a wide variety of crops.

Application of BTH effectively retarded the development of *D. rosae*. It was shown that BTH treatment did not have effect on conidia germination of *D. rosae*. Inconsistent results were obtained in previous investigation of the resistance mechanism to *D. rosae*. Conidia of *D. rosae* failed to germinate on the leaves of blackspot-resistant rose species (Reddy et al. 1992). Recently, Wiggers et al. (1997) reported that the rates of conidial germination on resistant cultivars were nearly the same as on the susceptible ones. One possible explanation for the conflicting results was due to the differences in responses of different rose plants to different sources of *D. rosae*. The experiments in the present study provided the evidence that the BTH-induced resistance did not result from a reduction of the percentage germination of conidia on treated rose leaves. According to Xue and Davidson (1998), leaf area with symptoms and sporulation capacity were two important components of resistance to blackspot in rose. The disease severity of blackspot in the experimental conditions was significantly reduced in the BTH-pretreated leaves (Figure 3.1 and Plate 3.6). The degree of reduction was dependent on the BTH concentrations. With the help of trypan blue staining, it was found that hyphal growth was restricted in BTH-pretreated leaves at an early stage of post-inoculation (Plate 3.7). By 7 days after inoculation, the infected leaves of control (without BTH pretreatment) showed many sporulating colonies (Plate 3.8C). In contrast, very few sporulating colonies were found on the

BTH-pretreated leaves (Plate 3.8D). The responses of BTH-induced resistance to *D. rosae* were consistent with observations on blackspot resistant cultivars (Xue and Davidson 1998). In summary, BTH treatment did not seem to have the effect on conidia germination of *D. rosae* but somehow later the development and severity of blackspot disease was modulated by mechanisms mediated by BTH. A similar observation has been made in transgenic rose plants expressing a rice chitinase gene with an increased resistance to *D. rosae* (Marchant et al. 1998).

The reduction in the frequency of crown gall formation and gall sizes in BTH pretreated rose shoots strongly suggests that BTH can also induce resistance to *A. tumefaciens* in rose. *Arabidopsis* plants developed resistance to *Pseudomonas syringae* and bean plants became resistant to *Xanthomonas campestris* pv. *Phaseoli* following BTH treatment, which seemed to restrict bacterial growth (Lawton et al. 1996, Siegrist et al. 1997). Thus, BTH-induced resistance to *A. tumefaciens* on rose shoots probably could also result from decreasing growth of the agrobacteria. However, exactly how BTH treatment could counteract *Agrobacterium*-mediated gall formation remains to be investigated.

4.1.3 The effect of BTH treatment on in vitro rose shoots

4.1.3.1 Effective concentration of BTH for inducing resistance to pathogens

In this study, BTH at a concentration as low as 50 μ M was sufficient to activate the resistance to *D. rosae* and *A. tumefaciens* in rose shoots. This compares favourably with other studies. For example, acquired resistance can be induced when exogenous application of BTH to grape at 8 mM (Wong et al. 1998), tomato and cucumber at 1.5 mM (Benhamou and Belanger 1998b, Benhamou and Belanger 1998a), tobacco at 1.2 mM (Friedrich et al. 1996), wheat at 1 mM (Görlach et al. 1996), and green bean at 0.1 mM (Siegrist et al. 1997). It could be argued that rose shoots grown *in vitro* are more sensitive to BTH stimulation compared with other crops in the previous studies, presumably because of the lack of waxy cuticles in the *in vitro* rose shoots. However, it was demonstrated previously that rose powdery mildew could be controlled by INA with a concentration of 1 mg/l, which is much lower than that used in other plant species (Ward et al. 1991b, Uknes et al. 1993, Dann and Deverall 1995). The results

in the present study also demonstrated that BTH could be autoclaved without losing its biological activity as far as induced resistance is concerned.

4.1.3.2 Phytotoxicity

Application with 50 μ M BTH to rose shoots significantly induced resistance to *D. rosae* and *A. tumefaciens* without visible symptoms of growth inhibition. However, the growth of the treated shoots was retarded and lower leaves turned yellow at later stage following the treatment with concentrations higher than 0.5 mM, indicating possible phytotoxic effects. The symptoms caused by phytotoxicity are consistent with the reports on other species (Siegrist et al. 1997, Godard et al. 1999, Tosi et al. 1999). However, this may be a property of *in vitro* grown shoots, often lacking surface protective cuticles, because spraying several weeks old rose pot plants with 0.5 mM BTH had no noticeable negative effects. Application of BTH at 5 mM occasionally brought about the formation of smaller, youngest leaves on pot rose plants. A similar result was observed that the youngest leaves of green bean were significantly affected by spraying higher than 0.4 mM BTH (Siegrist et al. 1997).

4.2 Characterisation of BTH-induced extracellular proteins

4.2.1 ICF extraction

In the present study, about 150-260 μ l of a yellowish extract were recovered from one gram of rose leaf tissue. The ICF extract contained about 0.5 mg of proteins per millilitre. It seems the yield and total protein content were lower than those of other plant species when ICF was extracted from leaves with the same extraction techniques (Parent and Asselin 1984, Kombrink et al. 1988). For example, in tobacco, approximately 0.5 ml ICF could be obtained from one gram fresh leaf tissue. The extract had a high concentration of protein with 1 mg of protein per millilitre (Parent and Asselin 1984). Meanwhile, the presence of cytoplasmic contamination was identified by assays of α -mannosidase activities in intra- and extracellular extracts from rose leaves. The results indicated that the ICF extraction procedure is efficient and reliable in rose.

It was also shown that there were no marked qualitative differences in the protein profiles of the ICFs extracted with either water or specific buffers. This result was consistent with previous observations (Parent and Asselin 1984, Tamás et al. 1997).

4.2.2 SDS-PAGE analysis of protein changes in the intercellular fluids of rose leaves

SDS-PAGE analysis revealed that BTH treatment induced the accumulation of at least ten different extracellular proteins in rose leaves, with molecular weights less than 40 kDa (Plate 3.13). It seems that the accumulation of proteins were dose-dependent. However, one 10 kDa band was only detected in 50 μ M BTH-treated leaves, while the 36 kDa protein was apparently present in 2.5 mM and 5 mM BTH-treated leaves. The 36 kDa protein started to accumulate at day 3 after the treatment with 5 mM BTH (Plate 3.15). Most of the BTH-induced proteins were also induced in response to blackspot infection.

The intensity of BTH-induced bands was more pronounced by dipping shoots into BTH solution than adding BTH in culture medium, probably because in the former there was a rapid and direct uptake and distribution of BTH throughout the leaves, whereas in the later the shoots without roots were less efficient in taking up BTH from the medium.

Most of BTH-induced proteins could be detected using the SDS-PAGE technique. However, some minor differences could not be observed due to the superimposition of two or more proteins, which could be more clearly displayed on 2D-PAGE gels.

4.2.3 2D-PAGE analysis of protein changes in the intercellular fluids of rose leaves

The time courses of the accumulation of extracellular proteins in BTH-treated and control leaves were investigated by 2D-PAGE analyses. Compared to SDS-PAGE, 2D-PAGE of intercellular fluid extracts was more efficient in displaying the difference in the protein patterns between water-treated and BTH-treated leaves over

the period of 7 days. BTH treatment induced the appearance of 7 new proteins and increased the abundance of 7 others (Table 3.4).

The expression patterns of extracellular proteins in response to BTH treatment were very similar to those for blackspot infection displayed on 2D-PAGE. Twelve extracellular proteins induced by BTH treatment were also found in the infected leaves by *D. rosae* (compare Table 3.4 with Table 3.6). Only two other proteins (No. 17 and 18) seemed to be specially induced by BTH. Further studies are needed to purify them, obtain N-terminal amino acid sequencing data, isolate and characterise their genes and assess their potential roles in disease resistance. The accumulation of 28 kDa (No. 12), 36 kDa (No. 15), and 37 kDa (No. 16) proteins could occur as early as 1 day after BTH treatment. Thus, these proteins might be used as early indicators of BTH-induced resistance in rose.

4.2.4 Western blot analysis of protein changes in the intercellular fluids of rose leaves

The polyclonal antibodies, produced against the intercellular fluid extracted from the leaves 5 days after BTH treatment, recognised a number of extracellular proteins by immunoblotting analysis. After the adsorption procedure, the antiserum detected 8 bands from the extract of BTH-treated leaves, while only 3 bands were present in that of water-treated leaves (Plate 3.21B). Similar results were obtained by immunoblotting analysis against the polyclonal antibodies produced by the ICF from blackspot-infected leaves (Plate 3.22B). These results confirmed that BTH treatment activated a set of extracellular proteins in rose leaves.

In the future, these antisera obtained in the present study may be used to screen the λ gt 11 cDNA library or a similar expression library for isolation of the associated cDNA clones with rose PR proteins (Côté et al. 1991).

4.3 Characterisation of *D. rosae*-induced extracellular proteins

1.1.1 SDS-PAGE analysis of protein changes in the intercellular fluids of rose leaves

Changes in the pattern of extracellular proteins, associated with blackspot infection, were detected by SDS-PAGE (Plate 3.26). At least 10 proteins with molecular weights less than 40 kDa were found to accumulate strongly in infected leaves starting at day 3 after inoculation and increased in abundance with disease development.

4.3.2 2D-PAGE analysis of protein changes in the intercellular fluids of rose leaves

Two-dimensional gel electrophoresis is a very useful technique for analysis of protein changing patterns. It can display a large number of separated proteins and reveal protein changes that are not detected by SDS-PAGE or IEF. In this study, 2D gel electrophoresis was used to identify changes of extracellular proteins in leaves of rose shoots after inoculation with *D. rosae*. Sixteen proteins, which were absent or present at a low level in the healthy leaves, have been detected in the intercellular fluid extracted from the infected rose leaves 7 days after inoculation with *D. rosae* (Table 3.6). It can be seen that these proteins are of low molecular weights ranging from 14-37 kDa with acidic pIs.

A comparison of protein expression patterns of rose shoots inoculated with *D. rosae* versus shoots treated with BTH revealed that thirteen extracellular proteins were not only induced by the pathogen infection, but also produced in the chemical-treated leaves. These results indicate that these PR proteins accumulated as a change in gene expression in the host plant. However, three proteins were detected only in the pathogen-infected rose leaves. They were not induced by the BTH treatment. Probably, these proteins are specifically induced by *D. rosae*, but it cannot be ruled out that they might be fungal proteins.

4.3.3 Immunological detection of extracellular PR proteins

It is interesting to find a close serological relationship between rose PR proteins and tobacco PR proteins, based on their kinetic accumulation against antisera of the four major classes of tobacco PR proteins (Plate 3.29 and Plate 3.30). Compared with tobacco extracellular PR proteins, a large number of acidic PR proteins were also found in the intercellular fluids of rose leaves after infection with *D. rosae*. However, the tobacco and rose PR proteins have different molecular weights and isoelectric points.

The antiserum of tobacco PR-1 proteins, which were identified in many plants upon infection by a pathogen or by treatment with chemicals (Linthorst 1991), cross-reacted with the rose 15 kDa protein. The molecular weight of this protein is similar to that of tobacco (approximately 15 kDa). It accumulated at later stages of the pathogen infection, but undetected in systemic leaves, i.e. newly expanded and uninfected leaves. Whether the other two extracellular proteins (27 and 38 kDa) recognised by the tobacco anti-PR-1c are all PR-1 proteins needs to be studied in future experiments as the limited supply of this antiserum of tobacco PR-1c protein was run out.

In healthy leaves of rose shoots, PR-2 (36 kDa), PR-3 (25 and 33 kDa) proteins were expressed at low levels, and PR-1 (15 kDa), PR-2 (26 and 37 kDa), PR-3 (37.5 kDa), and PR-5 (25.5 and 27.5 kDa) proteins were undetectable. In contrast, accumulation of three PR-2, three PR-3 and two PR-5 proteins was detected in infected rose leaves during blackspot development as well as in the systemic leaves. However, the 15 kDa PR-1 protein was only detected in the infected rose leaves at the later stage of infection.

Taken together, the results of this study demonstrated that *D. rosae* infection led to the coordinated induction of PR-1, PR-2, PR-3 and PR-5 proteins in infected rose leaves and PR-2, PR-3 and PR-5 proteins in the upper uninfected systemic leaves.

The accumulation of PR proteins in leaves is strongly related to plant disease resistance (Bol et al. 1990, Linthorst 1991). In particular, it seems advantageous to accumulate PR proteins in the intercellular spaces which can form initially cellular barriers against invading pathogens (Mauch and Staehelin 1989). It has been suggested that they may play an important role in plant defence response. However, whether the expression of these defence genes in systemic leaves can effectively protect rose plants from pathogen infection requires further investigation. As far as we know, this is the first investigation on the induction and characterisation of PR proteins in rose plants.

4.3.4 D. rosae-induced proteins displaying common characteristics of PR proteins

The induced proteins in rose by blackspot infection in this study share common properties of PR proteins in other plant species (Van Loon 1985, Bol et al. 1990, Linthorst 1991, Stintzi et al. 1993), which are inducible by a fungal pathogen, of low molecular weights (less than 40 kDa), found extracellularly, and have acidic isoelectric points and a close serological relationships with tobacco PR proteins of the same classes.

2D-PAGE analysis of ICF extract from blackspot-infected leaves revealed that the pathogen-induced proteins have acidic pIs. In tobacco, acidic PR proteins accumulate extracellularly while basic PR proteins accumulate intracellularly in the vacuole (Bol et al. 1990, Linthorst 1991, White and Antoniow 1991). It seems that rose extracellular PR proteins are similar to those of tobacco. However, some basic tomato and potato PR proteins were found to accumulate in the intercellular spaces of leaves as well (Linthorst 1991, White and Antoniow 1991).

4.4 Enzyme activities in the intercellular fluids of rose leaves

4.4.1 β -1,3-glucanase

The results indicate that BTH treatment led to an increase of β -1,3-glucanases in the intercellular spaces of rose leaves (Figure 3.4). The enzyme activity was increased

with the increasing concentrations of BTH. The induction and accumulation of β -1,3-glucanase also occurred in *D. rosae* infected leaves (Figure 3.10). However, the enzyme activity was much more rapidly and strongly enhanced in blackspot-infected leaves that were previously treated with BTH, suggesting that the increased β -1,3-glucanases may play a role in restricting the development of disease symptoms on the rose leaves infected with *D. rosae*. Since glucan is a major cell wall component of many plant pathogenic fungi, an antifungal role of β -1,3-glucanases has been proposed (Linthorst 1991, Stintzi et al. 1993). It was found that the induction and accumulation of β -1,3-glucanases were earlier and more pronounced in resistant cultivars than susceptible ones (Daugrois et al. 1990, Linthorst 1991, Stintzi et al. 1993, Kim and Hwang 1994, Roulin et al. 1997). Furthermore, some β -1,3-glucanases have been demonstrated to possess antifungal activity *in vitro* or *in vivo* (Mauch et al. 1988, Sticher et al. 1997). In addition, it has been suggested that β -1,3-glucanases may also be involved in the stimulation of the plant defence reaction by releasing oligosaccharides that can induce phytoalexin production in host cells (Mauch and Staehelin 1989, Takeuchi et al. 1990). These data further support the hypothesis that β -1,3-glucanase may play a role in plant disease resistance.

Detection of β -1,3-glucanases after native PAGE revealed the presence of two acidic extracellularly localised isoforms in rose leaves (Plate 3.34 and 3.35). One of the isoforms (G1) with higher β -1,3-glucanase activity of pI 5.7 was strongly induced following the treatment with BTH. It was detected in basic gel 3 days after BTH treatment and increased with times. In addition, this isoform was expressed at a much higher level in BTH-pretreated leaves 3 days after challenge inoculation with *D. rosae* (Plate 3.44). These results suggest that this β -1,3-glucanase isoform may be involved in the resistance to *D. rosae* in rose. The isoform (G2) was found to express strongly in senescent leaves as well as water-treated leaves of *in vitro* rose shoots, suggesting that this β -1,3-glucanase isoform could be developmentally regulated as reported previously in other plants (Cordero et al. 1994, Vögeli-Lange et al. 1994).

The induction of β -1,3-glucanase activity in the intercellular spaces of rose leaves by BTH was further confirmed by western blotting against tobacco anti-PR-N (Plate 3.23). However, it was surprising to note that three PR-2 bands with different molecular weights were detected on the immunoblots, while two bands with the enzyme activity were found on the nondenaturing gels. Meanwhile, only one strong band with β -1,3-glucanase activity was detected in the ICF extract from senescent leaves on the native PAGE (Plate 3.36), whereas two bands (26 and 36 kDa) were detected on the western blot (Plate 3.46). The possible explanations for this phenomenon were that the charge to weight ratio of the two proteins with β -1,3-glucanase activity is very similar. In addition, the two extracellular enzymes may tend to aggregate together under nondenaturing conditions or the two isoforms may have a similar three-dimensional structure. This result is similar to the previous observation showing that the bifunctional chitinase/lysozyme and β -1,3-glucanase comigrate together under nondenaturing conditions and can be separated by isoelectric points in cucumber (Ji and Kuc 1995).

4.4.2 Chitinase

The BTH treatment induced the synthesis and accumulation of chitinases in the intercellular spaces of rose leaves (Figure 3.6). The enhanced activity of chitinase by BTH treatment was also found in cucumber (Kästner et al. 1998), green bean (Siegrist et al. 1997), tomato (Inbar et al. 1998), sugar beet (Braun-Kiewnick et al. 1998a) and barley (Braun-Kiewnick et al. 1998b). Induction of this enzyme was more distinctly in BTH-pretreated leaves than in water-pretreated leaves after inoculation with *D. rosae* (Figure 3.11).

Staining of chitinases after native PAGE revealed that two acidic extracellular chitinase isoforms (C1 and C2) were induced by BTH treatment (Plate 3.38). Meanwhile, it was found that BTH stimulated the activity of the same set of chitinase isoforms as did the *D. rosae* in rose leaves, but the isoforms (C3 and C4) were more strongly induced and accumulated in *D. rosae* infected leaves previously treated with BTH (Plate 3.45).

Chitinases and β -1,3-glucanases have shown to possess antifungal activity alone or in combination (Mauch et al. 1988, Verburg and Huynh 1991, Huynh et al. 1992, Zhu et al. 1994). It has been suggested that extracellular chitinases and β -1,3-glucanases would have an initial role in restricting fungal growth in induced plants (Mauch et al. 1988, Mauch and Staehelin 1989). Thus, in the present study, higher levels of β -1,3-glucanase and chitinase activity were present in the intercellular spaces of BTH-treated rose leaves before challenge inoculation when compared with the water-treated leaves and accumulation of the two hydrolases was much more pronounced in *D. rosae* infected leaves previously treated with BTH. The activities of both enzymes were increased with the increasing concentrations of BTH applied in rose shoots, while the disease severity of blackspot in the experimental conditions was reduced with the increasing BTH concentrations. Taken together, these results strongly suggest that β -1,3-glucanases and chitinases are involved in rose disease resistance.

4.4.3 Peroxidase

The data obtained from the literature indicate that induced disease resistance is associated with enhanced levels of extracellular peroxidase in a range of plant species in response to pathogen attack or chemical treatment (Smith et al. 1991, Miyazawa et al. 1998). Increased peroxidase activity associated with BTH-induced resistance was demonstrated in green bean (Siegrist et al. 1997) and tomato (Inbar et al. 1998). The possible role of peroxidases in plant defence response has been proposed, for example, in increasing lignification, cross-linking of extension monomers and oxidised phenolic compounds, which all could contribute to disease resistance. But, direct evidence has not yet been obtained as transgenic plants overexpressing a tobacco anionic peroxidase gene in tomato or a cucumber anionic peroxidase gene in potato did not result in enhancement of resistance to pathogen (Lagrimini et al. 1993, Ray et al. 1998). It was also found that all isoforms of peroxidase did not seem to be associated with disease resistance. For example, a number of extracellular peroxidase isoforms existed in barley, but only two isoforms showed an increasing expression of peroxidase activity following inoculation with *Erysiphe graminis* f. sp. *hordei* (Kerby and Somerville 1989). The results in the present study indicate that BTH treatment

stimulated an increased expression of one acidic and one basic isoform in rose leaves. The induction of peroxidase activity in BTH-treated leaves was consistently associated with the induction of chitinase and β -1,3-glucanase. However, peroxidase activity in BTH-treated leaves did not increase beyond 5 days after the chemical treatment (Figure 3.9). In addition, infection with blackspot did not lead to an increase in the activity of peroxidase in the leaves treated with or without BTH within the experimental period. Therefore, the possible significance of increased peroxidase activity in rose disease resistance requires further investigation.

4.5 The BTH-induced disease resistance is associated with the induction and accumulation of extracellular PR proteins

Exogenous application of BTH to tobacco and *Arabidopsis* leaves has resulted in the coordinate induction of a set of PR genes that are termed SAR genes, leading to an enhanced resistance to various pathogens (Friedrich et al. 1996, Lawton et al. 1996). Therefore, major efforts in the present study have been focused on PR proteins of possible involvement of the same kind of resistance mechanism to pathogens in rose.

It was shown that most extracellular proteins activated by the BTH treatment (Table 3.4) were also induced and accumulated in the leaves after inoculation with *D. rosae* (Table 3.6). The induction of PR proteins were further confirmed by immunological analysis using antisera raised against several tobacco PR proteins. The properties of the rose extracellular PR proteins induced by BTH treatment are summarised in Table 3.5.

It is interesting to note that three bands with different molecular weights presenting in the intercellular fluids of rose leaves were recognised by tobacco anti-PR-1c in response to BTH treatment and blackspot infection. The 15 kDa PR-1 protein was detected as one band in 15% SDS-PAGE gels, but two spots with similar molecular weights induced by BTH and blackspot infection were detected in 2D-PAGE gels. Thus, further investigation is needed to clarify whether the two proteins all belong to the group of PR-1 proteins. In addition, two additional bands with

molecular weights about 27 and 38 kDa are probably PR-1-like proteins or they exhibited unspecific binding with the antiserum.

In the present study, the 15 kDa PR-1 protein was induced by BTH treatment. In particular, this protein rapidly accumulated to a much higher level in BTH-pretreated leaves upon infection with *D. rosae* (Plate 3.31). This suggests that the accumulation of PR-1 protein (15 kDa) may be involved in resistance to *D. rosae* in rose. A strong induction of PR-1 proteins after BTH treatment has also been demonstrated in tobacco (Friedrich et al. 1996), *Arabidopsis* (Lawton et al. 1996), maize (Morris et al. 1998) and wheat (Görlach et al. 1996). Much published data has indicated that the PR-1 proteins are the most common and abundant proteins induced by pathogen attack or by chemicals (Linthorst 1991, Stintzi et al. 1993). They are often used as molecular markers for SAR as their expression is tightly correlated with the establishment of disease resistance. It is assumed that they play a role in acquired disease resistance in plants (Ward et al. 1991b, Vernooij et al. 1995). Thus, it is also likely that the PR-1 protein (15 kDa) might be a marker of disease resistance in rose.

PR-2 proteins in the intercellular spaces of rose leaves were markedly induced following BTH treatment. Furthermore, upon challenge inoculation with *D. rosae*, the 36 and 37 kDa PR-2 isoforms were significantly induced again, and more rapidly and strongly in BTH-pretreated leaves than in water-pretreated leaves (Plate 3.31), indicating that these specific enzymes may play an important role in the rose disease defence. The marked increase in β -1,3-glucanase activity in connection with disease resistance was also observed in BTH-treated leaves of green bean (Siegrist et al. 1997), sugar beet (Burketová et al. 1999), tomato (Inbar et al. 1998), barley (Braun-Kiewnick et al. 1998b) and cotton plants (Colson-Hanks and Deverall 2000).

PR-3 proteins were also induced by BTH treatment. The 37.5 kDa isoform was found to accumulate at day 4 after BTH treatment, while the 25 kDa and 33 kDa PR-3 proteins accumulated gradually in BTH-treated leaves. Upon challenge inoculation with *D. rosae*, the 33 kDa isoform seemed to respond more quickly and strongly than other members of the same family in BTH-pretreated leaves of rose shoots, suggesting these isoforms may be also involved in rose defence response. In addition, it is

interesting to find that three bands were detected on immunoblots against tobacco PR-Q (Plate 3.24 and 3.32), while four spots were detected on 2D-immunoblots (Plate 3.25B), i.e. two PR-3 protein isoforms with the same molecular weights (37.5 kDa) are of different pIs. This result was further confirmed by detection of chitinases after native PAGE. However, the two PR-3 spots identified on 2D-immunoblots were undetected on silver stained 2D-PAGE gels or from isozyme assay on IEF gels. This is probably because the amount of the two proteins induced by BTH treatment was relatively scarce in the intercellular fluids compared with other induced proteins.

It has been reported that the combinations of β -1,3-glucanases and chitinases more effectively inhibited fungal growth than either enzyme alone (Mauch et al. 1988). In the present studies, PR-2 and PR-3 proteins were coordinately induced in BTH-treated leaves and become much more pronounced in BTH-pretreated leaves following inoculation with *D. rosae*, suggesting that these proteins may inhibit the hyphal growth of *D. rosae*. However, not all the isoforms of the two enzymes seemed to be associated with disease resistance in rose.

PR-5 genes were induced by BTH treatment in tobacco (Friedrich et al. 1996), *Arabidopsis* (Lawton et al. 1996), and maize (Morris et al. 1998). The rose extracellular PR-5 proteins were also induced by this chemical (Plate 3.24). Particularly, these proteins were expressed strongly in BTH-pretreated leaves after infection with *D. rosae* (Plate 3.32). These results showed that expression of PR-5 proteins is associated with disease resistance in rose.

The extracellular PR-1, PR-2, PR-3 and PR-5 proteins were coordinately induced and started to accumulate around 3 days after BTH treatment, reaching maximal levels at day 5-7. Thus, basing on the time course of accumulation of these proteins, the time period required for BTH to induce disease resistance in rose is about 4 days.

PR-1 proteins in the intercellular spaces of tobacco leaves are the most predominantly induced PR proteins upon infection by viruses, fungi or chemical treatments (Linthorst 1991, Stintzi et al. 1993). In the present study, it was demonstrated that extracellular PR-2, PR-3, and PR-5 proteins were induced more strongly than PR-1 proteins in response to BTH treatment or blackspot infection.

Taken together, all of these results provide evidence that the induced resistance by BTH in rose is associated with activation of a set of extracellular proteins including PR-1, PR-2, PR-3 and PR-5 proteins. To the best of our knowledge, this is the first report on the characterisation of rose extracellular proteins, which were correlated with disease resistance induced by BTH.

4.6 The induction and regulation of the four major classes of extracellular PR proteins by other inducers

It is clear that some PR proteins are induced in response to pathogen attack or chemical treatment in a broad range of plant species (Carr and Klessig 1989, Bol et al. 1990, Bowles 1990, Linthorst 1991, Stintzi et al. 1993). In the present study, in addition to BTH treatment and blackspot infection, several other chemicals and leaf senescence also stimulated the accumulation of extracellular PR proteins in rose leaves (Plate 3.46 and Plate 3.47). However, there are some differences in induction and expression of different isoforms of PR proteins in rose leaves brought about by the factors studied here.

It is of interest to note that some differences in the expression of PR proteins were found between the extracts from the leaves of shoots grown *in vitro* and the leaves of potted plants in soil. Some isoforms of PR proteins such as 27 and 38 kDa PR-1, 26 and 36 kDa PR-2, and 25 and 33 kDa PR-3 proteins were not only induced by the different treatments, but were also present in leaves of control shoots cultured on the shoot growth medium for 5 days after transferring from multiplication medium containing BA, GA and NAA. It has been reported that exogenous application of plant hormones affected the expression of genes encoding PR proteins (Shinshi et al. 1987, Grosset et al. 1990). For example, PR-1 and PR-3 isoforms were found to accumulate in tobacco shoots after application of kinetin (Memelink et al. 1987). However, it was also reported that chitinase accumulation can be blocked by auxin and cytokinin in cultured tobacco tissues (Shinshi et al. 1987).

Rose PR-2, PR-3 and PR-5 proteins were slightly induced by mercuric chloride, but no PR-1 proteins were detected after treatment with the chemical. The

concentration of mercuric chloride applied to rose shoots were probably too high and had a strong phytotoxic effect. Similar results were obtained in sunflower without detectable accumulation of PR-1 and PR-2 proteins (Jung et al. 1995). In soybean, mercuric chloride treatment led to disappearance of one β -1,3-glucanase isoform which was constitutively expressed in hypocotyls and leaves (Yi and Hwang 1996).

Exogenously applied 1.5 mM SA to rose shoots only induced the expression of PR-2, PR-3 and PR-5 proteins, but not the 15 kDa PR-1 protein. However, the exogenous application of SA can effectively induce the expression of PR-1 genes and disease resistance in tobacco (Ward et al. 1991b), cucumber (Métraux et al. 1990), and tomato (Spletzer and Enyedi 1999). It is possible that the SA concentration tested is too low to activate the expression of PR-1 gene or as the work on tomato has shown foliar application of SA is not an effective method for its delivery to the leaf interior (Van Kan et al. 1995).

In addition, it is worthy to note that ethephon and senescence induced the same PR proteins with the same intensity, suggesting that the senescence-induced expression of PR proteins seemed to be mediated by ethylene. Rose PR-1 protein (15 kDa), which was absent in water-treated leaves, were detected in leaves treated with ethephon, BTH and pathogen infection as well as senescent leaves, suggesting that this protein may not be only involved in plant defence response, but also under developmental regulation. This result is in agreement with the investigation of Henriquez & Sanger (1982). The expression of PR-1 genes in tobacco was also regulated developmentally (Lotan et al. 1989). In addition, *Brassica napus* senescent leaves accumulated abundantly tobacco PR-1a type protein (Henriquez and Sanger 1982). Except the 15 kDa PR-1 protein, the 38 kDa PR-1-like protein was found accumulated in abundance in senescent leaves. This result is consistent with the findings of Vera and Conejero (1988), showing that P-69, an endoproteinase, was induced by citrus exocortis viroid in tomato leaves. It is also involved in normal ageing of the leaves and is probably an potential component of defence.

Although senescence of leaves is related to age, it is controlled by internal and external signals (Noodén et al. 1997). Recent studies have revealed that senescence is an active process requiring a change in gene expression (Smart 1994, Buchanan-Wollaston 1997, Gan and Amasino 1997). It was shown that ethylene plays a role in accelerating leaf senescence in some species (Mattoo and Aharoni 1988, Grbic and Bleecker 1995). Treatment with ethylene led to the accumulation of PR proteins as those induced after pathogen attack (Ishige et al. 1993a, Hanfrey et al. 1996). In the present study, most PR proteins induced by BTH and blackspot infection also accumulated in senescent leaves or in response to treatment with ethephon (an ethylene-releasing compound). Therefore, induction and expression of PR proteins in response to ethephon and senescence may have a common basis as a response against pathogen invasion. However, it cannot be excluded that these proteins are expressed as a result of developmental changes.

In light of the present results, it can be concluded, therefore, that the role of different isoforms of PR proteins may be different, some being involved in rose disease resistance while some having a role in the growth and development of plants as previously reported by workers (Fraser 1981, Lotan et al. 1989, Leung 1992, Smart 1994). Meanwhile, the results also indicated that BTH is a more effective inducer of PR protein expression than the other chemical inducers tested in the present study. Similar results were also found in sugar beet (Burketová et al. 1999), cucumber (Narusaka et al. 1999) and tobacco (Wendehenne et al. 1998).

4.7 The antifungal activity of BTH-induced extracellular proteins *in vitro*

It has been reported that most groups of PR proteins possess antifungal activity *in vitro* against a number of fungal pathogens (Schlumbaum et al. 1986, Mauch et al. 1988, Leah et al. 1991, Woloshuk et al. 1991, Niderman et al. 1995, Yun et al. 1996). However, the intercellular fluid from BTH-treated rose leaves did not seem to show any antifungal activity against *D. rosae* under the test conditions. This lack of antifungal activity is also reported in the crude ICF extract from induced cucumber (Ji

and Kuc 1996) and pea plants (Mauch et al. 1988). One possible explanation is that the intercellular fluid extracted from BTH-treated rose leaves might contain too low levels of specific PR proteins with antifungal activity. It is also possible that there might be some inhibitors against antifungal activity present in the ICFs. Thus, further experiments are needed to test whether the specific isoform from these PR protein families possess antimicrobial activity against different rose pathogens *in vitro*.

Chapter 5

GENERAL DISCUSSION

5.1 Involvement of extracellular PR proteins in BTH-induced rose disease resistance

BTH has been described as an efficient inducer of resistance to a wide spectrum of pathogens in many plant species in greenhouse as well as in field conditions (Friedrich et al. 1996, Dann et al. 1998, Inbar et al. 1998, Godard et al. 1999, Ishii et al. 1999, Colson-Hanks and Deverall 2000). Induction of resistance to *D. rosae* and *A. tumefaciens* was also demonstrated in rose following treatment with BTH. Disease development suppressed by BTH in rose is a result of activating the natural defence mechanisms rather than a consequence of directly inhibiting the growth and development of the pathogens by the chemical. It has been reported that BTH activates a number of PR genes which are mostly acidic extracellular proteins and their accumulation is tightly associated with disease resistance in a variety of plant species (Friedrich et al. 1996, Lawton et al. 1996, Siegrist et al. 1997, Burketová et al. 1999). Therefore, the mechanism of BTH-induced resistance in rose shoots has been investigated based on biochemical and immunological analyses of extracellular proteins, particularly, the expression of PR proteins.

It was demonstrated that application of BTH to rose leaves before challenge inoculation triggered a set of extracellular proteins, including PR-1, PR-2, PR-3 and PR-5 proteins, which were absent or present at a lower level in the water-treated leaves. Furthermore, upon inoculation with *D. rosae*, the BTH-pretreated shoots had a much greater expression of PR-1, PR-2, PR-3 and PR-5 proteins as compared with the

water-pretreated shoots. Particularly, the 15 kDa PR-1 protein, and 36 and 37 kDa PR-2 proteins seemed to respond more quickly and strongly than other PR proteins in BTH-pretreated leaves. These results suggest that BTH possibly further stimulate the accumulation of PR proteins in response to the pathogen infection.

The induction and accumulation of PR proteins by BTH may lead to the development of resistance following infection by pathogens in rose. This is possibly due to their antimicrobial actions as the PR-1, PR-2, PR-3 and PR-5 proteins of many other plant species are known to possess antimicrobial activities (Mauch et al. 1988, Roberts and Selitrennikoff 1990, Leah et al. 1991, Vigers et al. 1991, Liu et al. 1994, Zhu et al. 1994, Niderman et al. 1995).

PR-2 and PR-3 proteins possess β -1,3-glucanase and chitinase activities, respectively. The activities of both enzymes in the intercellular fluids were enhanced after BTH treatment and became more pronounced in BTH-pretreated rose leaves following inoculation with *D. rosae*. It has been demonstrated that β -1,3-glucanases and chitinases can function synergistically against fungal pathogens (Mauch et al. 1988, Sela-Buurlage et al. 1993). Thus, both enzymes could possibly be involved in the inhibition of the fungal infection in rose. However, not all the isoforms of these two enzymes seemed to be associated with disease resistance.

Taken together, these data may partly explain why BTH-pretreated shoots express an increased resistance to *D. rosae*. It also supports the hypothesis that extracellular PR proteins are involved in rose disease resistance. It has been suggested that coordinate expressions and interactions between multiple putative defence compounds in plants during pathogen infection are likely to be critical for resistance (Ward et al. 1991b, Ji and Kuc 1995). In tobacco and *Arabidopsis*, two model plants for studying SAR, BTH induced the same set of PR genes as did biological inducers of SAR (Ward et al. 1991b, Uknes et al. 1992, Friedrich et al. 1996, Lawton et al. 1996). However, a biological model of SAR in rose has not been reported. Therefore, it is not clear at present whether the proteins induced by BTH are also systemically accumulated by biological inducers.

5.2 Mechanisms of BTH action

The broad-spectrum activity of BTH, providing protection against viral, bacterial, and fungal diseases, even reduction of insect density, strongly suggests an indirect mode of action via activation of plant defence mechanisms. However, the mechanism of induced resistance in plants by BTH is still not completely elucidated. It has been proposed that BTH activates plant defence responses as it is translocated throughout the plant, acting either directly or through intermediates, inducing the expression of PR genes at much high levels at untreated parts of plants. It was found that BTH is most likely to act at a site downstream of SA accumulation in the defence mechanism (Friedrich et al. 1996, Lawton et al. 1996) and as a strong inhibitor of catalase and ascorbate peroxidase (Wendehenne et al. 1998).

The induction and expression of PR genes after BTH treatment, both at the mRNA and at the protein level, associated with induced disease resistance has been reported in a number of plant species (Friedrich et al. 1996, Görlach et al. 1996, Lawton et al. 1996, Siegrist et al. 1997, Inbar et al. 1998, Morris et al. 1998). Our results support the hypothesis that accumulation of PR proteins prior to challenge may play an important role in plant defence response. However, antiviral activity has not been found for any PR protein nor has enhanced resistance against viral pathogen from transgenic plants overexpressing PR genes. Thus, it is obvious that accumulation of PR proteins cannot fully account for the SAR broad-spectrum phenomenon. Therefore, it has been argued and proved that the resistance mechanism is not a single response but is rather the consequence of several defence reactions which are coordinated (Benhamou 1996, Kästner et al. 1998). For example, a set of new genes such as lipoxygenase and a sulphur-rich protein were also associated with resistance induced by BTH in wheat (Görlach et al. 1996). Recently, Katz et al. (1998) reported BTH has a dual role in the activation of plant defences in parsley cells. One group of defence genes such as SAR genes was directly responsive to BTH, whereas activation of another group of genes such as PAL gene was potentiated by pretreatment with BTH. This research emphasised an important role for defence response potentiation in acquired plant disease resistance. The conditioning phenomenon was also observed

in cucumber (Kästner et al. 1998). The acidic class III chitinase gene was expressed strongly upon a pathogen infection after the hypocotyls were pretreated with 2,6-dichlorisonicotinic (INA), salicylic acid (SA) or benzothiadiazole (BTH). The results in the present study seem to support this assumption as the induction and accumulation of extracellular PR proteins in rose were more pronounced in BTH-pretreated leaves than in water-pretreated leaves, in particular, PR-1 (15 kDa) and PR-2 (36 and 37 kDa) accumulated at much higher levels upon infection with *D. rosae*.

Cytological events occurring in BTH-induced plants could be also involved in restricting pathogen growth and development (Benhamou and Belanger 1998b, Benhamou and Belanger 1998a). For example, BTH-mediated induced resistance in tomato plants was accompanied by the rapid formation of callose-enriched wall appositions and activation of secondary response with antimicrobial activity such as synthesis of phenolic-like compounds at sites of potential fungal entry (Benhamou and Belanger 1998a). There are many kinds of phenolic compounds in plant cells. Some of them are involved in lignification and protection against infection (Hammerschmidt and Kuc 1982, Mäder and Amberg-Fisher 1982). The acquired resistance induced by BTH is associated with the massive accumulation of phenolic compounds in cucumber (Benhamou and Belanger 1998a) or synthesis of autofluorogenic compounds, probably of phenolic nature in wheat (Stadnik and Buchenauer 1999) at sites underlying pathogen penetration and the formation of structural barriers. It has been thought that phenolic compounds may play a key role in chemical-mediated induced resistance in cucumber by directly inhibiting fungal growth as a non-toxic glycosylated form of phenolic is converted into toxic aglycones (Benhamou and Belanger 1998b).

It has also been demonstrated that resistance mechanisms were activated more strongly in susceptible varieties than resistant ones, suggesting that BTH may stimulate inherent defence mechanisms so that the plants can respond more quickly against pathogen attack (Dann et al. 1998).

In summary, effective resistance is likely the result of a coordinated action of several defence reactions (Benhamou 1996). However, similarities and differences on defence mechanisms among plant species may exist (Benhamou and Belanger 1998a).

5.3 Future work

As shown here, roses can acquire resistance to fungal (*D. rosae*) and bacterial (*A. tumefaciens*) pathogens following treatment with BTH. It is likely that BTH may also induce resistance to a viral pathogen such as prunus necrotic ringspot virus (PNRV) as BTH has been shown to induce a broad-spectrum resistance in a number of plant species (Friedrich et al. 1996, Görlach et al. 1996, Siegrist et al. 1997, Inbar et al. 1998).

BTH has a promising effect on induced resistance against pathogens of roses *in vitro* under controlled environmental conditions. It is possible that the application of BTH as a novel approach will protect rose plants against diseases in the field. However, whether BTH can be applied at a low dose and only once to effectively control diseases, and whether rose plants can withstand environmental pressures under commercial production conditions will need to be investigated further. Up to now, systemically induced resistance has been demonstrated under field conditions for a limited number of plant/pathogen interactions (Görlach et al. 1996, Dann et al. 1998). Single BTH application to wheat and rice induced long-lasting resistance against fungal diseases (Görlach et al. 1996). However, in several crops such as soybean (Dann et al. 1998) and tobacco (Cole 1999), multiple BTH applications were necessary to induce and maintain disease resistance in both field and greenhouse conditions.

The biochemical events associated with disease resistance mechanisms have been investigated in this study, mainly on the expression of extracellular PR proteins after BTH treatment. Further work is needed to investigate whether the intracellular PR proteins are also involved in the mechanism of rose disease resistance induced by BTH. Certainly, in other work, it has been proposed that the intracellular PR proteins may play a role in the later infection process when cell breakage releases vacuolar

contents into the extracellular spaces (Mauch and Staehelin 1989, Steicher et al. 1992). Further studies are also necessary to investigate the systemic expression patterns of PR genes at the mRNA level after BTH treatment or inoculation with a pathogen to determine which gene families are tightly associated with the onset of resistance. These results will provide information towards a better understanding of the molecular mechanisms of disease resistance in rose. In addition, the cytological events such as the accumulation of phenolic compounds, callose, and lignin-like materials that might take part in mediating resistance remain to be investigated.

In order to determine the potential role of the extracellular PR proteins identified in this study, it will be of great interest to purify them from rose plants and to test their antimicrobial activity *in vitro* or to isolate the genes encoding them. The genes, single or in various combinations, will be further engineered into plants to test whether transgenic rose plants constitutively expressing the appropriate gene constructs would have increased levels of resistance against rose pathogens. The approach of genetic engineering resulting in the improvement of disease resistance has been demonstrated in a variety of plant species (Broglie et al. 1991, Liu et al. 1994, Zhu et al. 1994, Tabei et al. 1998). Recently, Marchant et al. (1998) reported that the transgenic rose plants expressing a rice chitinase gene exhibited less severe infection with blackspot. Nevertheless, it remains to be seen if transgenic rose plants expressing PR genes isolated from roses may be more effective in suppressing disease development.

In conclusion, the findings reported here should contribute toward the development of biotechnological approaches in disease protection in rose.

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APPENDICES

Appendix 1 MS Medium (Murashige & Skoog, 1962)

1 Litre

Major salts (10×)	NH ₄ NO ₃	16.5 g
	KNO ₃	19.0 g
	CaCl ₂ ·2H ₂ O	4.4 g
	MgSO ₄ ·7H ₂ O	3.7 g
	KH ₂ PO ₄	1.7 g
Minor salts (100×)	KI	0.083 g
	H ₃ BO ₃	0.620 g
	MnSO ₄ ·4H ₂ O	2.230 g
	ZnSO ₄ ·7H ₂ O	0.860 g
	CuSO ₄ ·5H ₂ O	0.0025 g
	CoCl ₂ ·6H ₂ O	0.0025 g
	Na ₂ MoO ₄ ·2H ₂ O	0.025 g
Organic supplement (100×)	myo-inositol	10 g
	nicotinic acid	50 mg
	pyridoxine-HCl	50 mg
	thiamine-HCl	10 mg
	glycine	200 mg
Iron stock (100×)	FeSO ₄ ·7H ₂ O	2.78 g
	N ₂ EDTA·2H ₂ O	3.73 g

Store at 4°C

Appendix 2 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)**Separating and stacking gel preparation**

Solution for Tris/Glycine SDS-PAGE	15% Separating gel	4% Stacking gel
	(ml)	(ml)
dH ₂ O	3	7.5
1M Tris-HCl (pH 8.8)	11.25	
1M Tris-HCl (pH 6.8)		1.25
30% Acrylamide:bis	15	1
10% SDS	0.3	0.1
10% Ammonium persulphate (APS)	0.3	0.04
TEMED	0.015	0.015

Electrophoresis buffer (1 litre)

	(g)
Tris Base	4.54
Glycine	21.6
SDS	1.5

Sample buffer (5 ×)

	10 ml
1 M Tris-HCl (pH 6.8)	0.6
50% Glycerol	5
10% SDS	2
2-mercaptoethanol	0.5
1% Bromophenol blue	1
dH ₂ O	0.9

Coomassie Blue gel staining

	1 Litre
Coomassie Blue R-250	1 g
Methanol	500 ml
Glacial acetic acid	100 ml
dH ₂ O	400 ml

Coomassie Blue gel destaining

	1 Litre
Methanol	50 ml
Glacial acetic acid	100 ml
dH ₂ O	850 ml

Appendix 3 Gel electrophoresis under non-denaturing conditions

High pH gel system for separation of acidic and neutral proteins

Gel preparation and electrophoresis running buffer are the same as SDS-PAGE, except that SDS was omitted from non-denaturing gels.

Sample buffer (5 ×) (Bollag et al. 1996a)

Solution	10 ml
1M Tris- HCl (pH 6.8)	3.1
Glycerol	5
1% Bromophenol blue	0.5
dH ₂ O	1.4

Low pH gel system for separation of basic proteins

Low pH gel preparation (8 ml)

	10% separating gel (ml)	4% stacking gel (ml)
Acetic acid-KOH pH 4.3	1	
Acetic acid-KOH pH 6.8		0.5
30% Acrylamide/Bis	2.67	0.53
dH ₂ O	4.33	2.97
10% Ammonium persulphate	0.06	0.02
TEMED	0.072	0.02

Appendix 4 Isoelectric focusing

Native gel sample buffer (2 ×)	5 ml
Pharmalyte 2D pH 3-10	40 µl
Resolyte pH 4-8	160 µl
Glycerol	3 ml
dH ₂ O	1.8

Native isoelectric focusing gel preparation	10 ml
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30% Acrylamide:bis	2 ml
Pharmalyte 2D pH 3-10	48 µl
Resolyte pH 4-8	240 µl
10% Ammonium persulphate	50 µl
TEMED	20 µl
dH ₂ O	7.7 ml

Appendix 5 Two-dimensional gel electrophoresis**Sample buffer (lysis buffer)**

	10 ml
Urea	4.8 g
CHAPS	0.2 g
β-mercaptoethanol	0.5 ml
Pharmalyte 2D pH 3-10	0.04 ml
Resolyte pH 4-8	0.16 ml

IEF gel preparation

Urea	10 g
Acrylamide/PDA (29.2/0.80)	2.5 ml
dH ₂ O	7 ml
Resolyte pH 4-8	0.8 ml
Pharmalyte 2D pH 3-10	0.2 ml
CHAPS	0.3 g
Nonidet P-40	0.1

Transfer buffer

	100 ml
0.5 M Tris-HCl (pH 6.8)	6.05 g
0.4% (w/v) SDS	0.4 g

2-D PAGE running lower buffer

	4 Litre
Glycine	57.6 g
Tris base	12 g
SDS	2 g
Sodium azide	0.4 g

2-D PAGE running upper buffer

	350 ml
Glycine	10.8 g
Tris base	2.1 g
SDS	0.35 g

Appendix 6 Western blotting

Towbin's transfer buffer

1 Litre

Tris 3.03 g

Glycine 14.4 g

Methanol 200 ml

* Store at 4°C

Blocking Solution

Dissolve 5 g of non-fat dry milk in 50 ml of TBS. Bring volume to 100 ml with TBS.

This solution should be prepared freshly.

10 × TBS

1 Litre

Tris base 12.11 g

NaCl 87.66 g

1N HCl 39 ml

* Adjust pH to 7.5 with 1N HCl

NBT Stock

Dissolve 0.5 g of NBT (nitro blue tetrazolium) in 10 ml of 70% dimethylformamide and store at 4°C.

BCIP Stock

Dissolve 0.5 g of BCIP (bromochloroindolyl phosphate p-toluidine salt) in 10 ml of 100% dimethylformamide and store at 4°C.

Alkaline Phosphatase Buffer**1 litre**

Tris base 12.11 g

NaCl 5.84 g

MgCl₂ • 6H₂O 1.02 g

* Adjust pH to 9.5; Store at 4°C

Appendix 7 Reports of N-terminal sequence analysis

Sample (spot on electroblot)	Sample applied	Sequence obtained	Comments
No. 9	PVDF wash: 1 × MeOH, 0.1% TEA and 2 × MeOH	X R F D	Convincing sequence for residues 2-4, but yield too low (≈0.5 pmol) to enable a clear call for residues.
No. 15	PVDF wash: 1 × MeOH, 0.1% TEA and 2 × MeOH	X K I ? V	Not very convincing sequence, yield about 0.2 pmol. Suggest repeating with at least 4 gel spots electroblotted
No. 16	PVDF wash: 1 × MeOH, 0.1% TEA and 2 × MeOH	Not convincing	Suggest repeating with at least 6 gel spots electroblotted

The N-terminal sequence determination of the blotted proteins was carried out in a Protein Microchemistry Facility by Ms Diana Carne, Department of Biochemistry, University of Otago, New Zealand.